Genetic Diversity and Regenerative Potential of *Tilia cordata* Miller in the Lincolnshire Limewoods

Amanda Julie Mylett

A thesis submitted in partial fulfilment of the requirements of the University of Lincoln for the degree of Doctor of Philosophy

August 2015

Abstract

The Lincolnshire Limewoods are a group of Ancient Semi Natural Woodlands within Central Lincolnshire that include the nationally important Bardney Limewoods National Nature Reserve. The woods, although fragmented and isolated by tracts of agricultural land, are reservoirs of biodiversity and contain large populations of *Tilia cordata* Mill. The current management aims are to increase the biodiversity within the woods, as well as to extend and improve the connectivity between the woodlands, with new planting. An understanding of the genetic diversity and structure of the Limewoods, both as individual woods and by comparison with woods from other regions of Britain, will help to inform management decisions.

A pilot study was undertaken using RAPD markers which demonstrated the potential for these markers to amplify and identify individual *T. cordata* trees. Dominant markers are less informative than co-dominant markers, especially when trees may be closely related, and to facilitate this study a *T. cordata* enriched microsatellite library was constructed. The ten microsatellite loci designed for the genetic study amplified both *T. cordata* and closely related *Tilia platyphyllos* Scop. and were also able to identify hybridisation between the two species.

T. platyphyllos and hybrid trees were detected in eleven of the Lincolnshire Limewoods and were associated with identification of private alleles within the *T. cordata* populations. The high levels of genetic diversity and low genetic variance which were found show that the Lincolnshire Limewoods' populations are all similar. Comparison with populations from outside Lincolnshire show similar genetic diversity, with AMOVA

conducted over all populations showing that only 4% of the variation could be allocated between the populations whereas 82% was allocated between the individuals within the populations. Weak isolation by distance was identified and would suggest that the Lincolnshire Limewoods should be treated as a single population group for management purposes. To increase the likelihood that *T. cordata* is used for replanting schemes, seeds should be taken from woods without hybrid populations and, preferably, should be screened to identify and exclude F1 hybrids.

Tissue culture was considered as a potential source of planting stock for the Lincolnshire Limewoods to provide trees of known provenance with potentially rejuvenated characteristics. Preliminary tissue culture investigation showed that the initial treatment and age of the tissue before sterilisation is important in controlling contamination after sterilisation. Tissue collected in the spring, prior to leaf emergence, with the buds allowed to develop under clean laboratory conditions resulted in a reduction in the number of contaminated explants. In explant culture, roots were induced with application of the auxin naphthaleneacetic acid (NAA) and microshoots were induced with a combination of NAA and cytokinin, 6-benzyl-aminopurine (BAP). However, no rooted plantlets were produced. Further investigation into the use of somatic embryos as an explant source should now be considered as a possible way of reducing the chronic contamination that was experienced using axial buds.

Acknowledgements

I would like to thank my supervisors, Dr C. Casey, Dr R. Dixon and Dr S. Brown. I am particularly grateful to Dr Sarah Brown who has been with me throughout this journey, providing help, support, constant encouragement and friendship. Also Dr Ciara Casey, who has been a friend and a mentor.

I would like to thank my work colleagues Dr Taghread Hudaib, Dr. Bill Hayes and Rachel Farrow for their support over the years. I am also grateful for the help I received from Anne Goodall, who advised me on all aspects of the Bardney Limewoods, and to members of the Limewoods Working Group, especially Hugh Milner and Rodger Clooney, who collected samples for this study.

My thanks go to my children Ian, Joanna, Katie and my sister, who have encouraged me throughout, but especially to my husband Dave, who, from collecting samples to fixing computers, has always been there to help.

Thanks must also go to the Forestry Commission, especially Wally Grice, as well as the Lincolnshire Limewoods Project and Liz Fleuty for facilitating access to and providing information about the woods.

I would also like to recognise that this research would not have been possible without the support of the Department of Biological Science at the University of Lincoln.

CONTENTS

Abstract i
Acknowledgements iii
Contents iv
Figures ix
Tables xv
Glossary of Abbreviations xviii

CHAPTER ONE
General Introduction 1

1.1 Introduction 2
1.2 The Bardney Limewoods 3
1.3 Ancient and Semi-Natural Woodland 8
1.4 The Origin of Ancient Limewoods in Britain 9
1.5 The Characteristics of *Tilia cordata* Miller 13
1.6 Regeneration of *Tilia cordata* within the Limewoods 23
1.7 The study of genetic variation and structure in *Tilia* spp. 28
1.8 Aims of the Study 31
1.9 References 33

CHAPTER TWO
Development of DNA Extraction Method and Initial Investigation into Genetic Diversity Using RAPD Markers. 41

2.0 Introduction 42

2.1 DNA Extraction 43
2.1.1 DNA Extraction Introduction 43
2.1.2 DNA Extraction Materials and Methods 45
2.1.2.1 Optimised Extraction Method Based on the CTAB B Protocol 48

2.1.3 DNA Extraction Results 51
2.1.4 DNA Extraction Discussion 54

2.2 RAPD Analysis 56
2.2.1 RAPD Analysis Introduction 56
2.2.2 RAPD Analysis Materials and Methods 57
2.2.3 RAPD Analysis Results 59
2.2.4 RAPD Analysis Discussion 63

2.3 References 65

CHAPTER THREE

The Development of Microsatellite Markers for the Investigation of the Population Structure of *Tilia cordata* Miller (Malvaceae) within the Lincolnshire Limewoods 69

3.1 Introduction 70

3.2 Materials and Methods 74
3.2.1 Microsatellite Library Development 75
3.2.2 Microsatellite Characterisation 84

3.3 Results 86
3.3.1 Genetic Diversity and Cross Amplification between Species 88

3.4 Discussion 94

3.5 References 98

CHAPTER FOUR

The Genetic Variation and Structure of *Tilia cordata* within the Lincolnshire Limewoods. 104

4.1 Introduction 105

4.2 Materials and Methods 109

4.2.1 Sample collection, DNA extraction and PCR 109

4.2.2 Identification of Clonal Ramets 114

4.2.3 Identification of *T. platyphyllos* and Occurrence of Hybridisation within the Population Groups 115

4.2.4 Identification of Population Genetic Diversity 117

4.2.5 Population Structure 118

4.2.5.1 Fine scale spatial genetic structure within local populations 122

4.3 Results 123

4.3.1 Identification of Clonal Ramets 123

4.3.2 Identification of *T. platyphyllos* and Hybridisation within the Populations 127

4.3.3 Genetic Diversity 133

4.3.4 Population structure 139

4.3.4.1 Cryptic Population Structure within the Limewoods 147

4.3.4.2 Fine Scale Spatial Genetic Structure 159

4.4 Discussion 161

4.4.1 Clonal Groups 161

4.4.2 Identification of *T. platyphyllos* among the *T. cordata* Populations and Hybridisation between *T. cordata* and *T. platyphyllos* 163

4.4.3 Genetic Diversity 166

4.4.4 Population Genetic Structure 169

4.4.5 Identification of Cryptic Population Structure 171

4.4.6 Fine Scale Spatial Genetic Structure 173

4.4.7 Conclusion 174

4.5 References 176

CHAPTER FIVE

Tissue Culture 188

5.1 Tissue Culture Introduction 189

5.1.1 Explant Selection 190

5.1.2 Aseptic Tissue Sterilisation 193

5.1.3 Tissue Culture Medium 194

5.1.4 Plant Growth Regulators 195

5.1.5 Aim 196

5.2 Tissue Culture Materials and Methods 197

5.2.1 Explant Selection, Preparation and Sterilisation 198

5.2.2 The comparison of two chlorine-based sterilisation solutions 201

5.2.3 Tissue Culture Medium and Plant Growth Regulators 203

5.3 Tissue Culture Results 205

5.3.1 Explant Selection, Preparation and Sterilisation 205

5.3.2 The Comparison of Two Chlorine-Based Sterilisation Solutions 207

5.3.3 Tissue Culture Medium and Plant Growth Regulators 214

5.4 Tissue Culture Discussion 219

5.5 References 228

CHAPTER SIX

General Discussion 235

6.1 Discussion 236

6.2 Further work 241

6.3 Conclusion 242

6.4 References 243

APPENDICES 247

Appendix 1 Maps of the General Location of all Woods Sampled and Maps of Individual Woods Showing Individual Trees with their Genotype Assignment. 248

Appendix 2 Pairwise Fst derived from AMOVA for: -
A. 20 wood populations with *T. cordata* trees
B. 21 wood populations with *T. cordata*, *T. platyphyllos* and hybrid trees. 270

Appendix 3 Tissue Culture Media Composition and Comparison 273

FIGURES

Figure 1.1 Map of the Lincolnshire Limewoods Project Area. This area includes the nine SSSI woods that comprise the Bardney Limewoods National Nature Reserves. 6

Figure 1.2 *T. cordata* together with *Hyacinthoides non-scripta* (L.) Rothm. and *Anemone nemorosa* L.; some of Lincolnshire's ancient woodland indicator plants (Scotgrove Wood spring 2011). 9

Figure 1.3 Isochrone map of the increasing extent of *Tilia* pollen in the British Isles over time. The isochrones estimate the date (years BP) of the palynological evidence of the presence of *Tilia* pollen (Birks, 1989). 12

Figure 1.4 The European distribution of *Tilia cordata* Mill (EUFORGEN, 2008a) and *Tilia platyphyllos* Scop.(EUFORGEN, 2008b). 15

Figure 1.5 Mature standard *T. cordata* in Poterhanworth Wood, Lincolnshire. 16

Figure 1.6 *T. cordata* coppiced stool less than 5 years old. 17

Figure 1.7 Four dominant trunks of a mature *T. cordata* coppiced tree. 17

Figure 1.8 Young red barked stems of *T. cordata* before leaves emerge. 17

Figure 1.9 A comparison between the abaxial leaf surfaces of *T. cordata* and *T. platyphyllos*. 18

Figure 1.10 *T. cordata* flowers held erect above the bracts above the leaf canopy. 19

Figure 1.11 Comparison between the erect flower bracts of *T. cordata* (left) and the pendulous bracts of *T. platyphyllos* (right). 19

Figure 1.12 *T. cordata* seedlings from Hatton Wood, Lincolnshire in May 2012. 20

Figure 1.13 Layered *T. cordata* coppiced stool from Great Scrubbs Wood, Lincolnshire. 26

Figure 1.14 Self-layered *T. cordata* branch; from Hardy Gang Wood, Lincolnshire. 27

Figure 1.15 Fallen *T. cordata* tree still growing and producing vertical shoots that may possibly root; from Skellingthorpe Old Wood, Lincolnshire. 27

Figure 1.16 Distribution and frequency of chloroplast DNA haploypes within European *Tilia cordata* populations **(**from Fineschi *et al*. (2003)). 30

Figure 2.1 The Comparison of four DNA extraction methods for *T. cordata*. Quantification of the genomic DNA extracted from approximately 260mg of *T. cordata* fresh leaf (10 µl loaded in each lane) can be estimated here by comparison with bands from 50 ng, 100 ng and 200 ng of lambda phage. Large amounts of RNA show up as bands with a size of < 500bp when measured against the included 100bp genomic ladder. All the samples were obtained from a single *T. cordata* tree from Great Scrubbs Wood in the Bardney Limewoods (individual tree identification Ch 2). 52

Figure 2.2 DNA extracted from *T. cordata*, using the modified CTAB B protocol, showing the effect of using RNase A to digest RNA. Genomic DNA was extracted from four separate trees taken from four of the Bardney Limewoods (Cocklode T (CLT), Wickenby G (WG), Hatton M (HM) and Southrey N (SN)). For individual trees, adjacent lanes compare DNA extracted with and without RNA removal. For the estimation of size and quantity of DNA extracted, lanes including 100 bp ladders and 50ng lambda phage DNA are also included. 53

Figure 2.3 A typical RAPD gel-banding pattern after amplification with primer 2 for *T. Cordata* from two woodland groups, Goslings Corner Wood (G) and Great Scrubbs Wood (Ch). HindIII cut lambda DNA (λ) is used as a molecular weight standard. 60

Figure 2.4 Cluster analysis, using Dice similarity coefficients, of the results from the RAPD analysis of *T. cordata*. The 12 trees were from two populations, eight trees from Great Scrubbs Wood (Ch) and four trees from Goslings Corner Wood (G). The numbers by the branches show the percentage of bootstrap analyses that support that branch (bootstrap values are derived from 100 replicates). 62

Figure 3.1 Summary of the microsatellite enrichment procedure. 76

Figure 3.2 The 1 % agarose gel image of the PCR product of the purified enriched fragments. Loading both 20 µl and 10 µl of the PCR product indicates that the fragments from the hybridisation and enrichment are mainly in the 500 to 300bp size range. 78

Figure 3.3 Summary of the cloning of restriction fragments enriched for microsatellites. 80

Figure 3.4 Cloning vector map for the pCR™ 2.1-TOPO® vector identifying the sequence surrounding the TOPO cloning site. The vector map indicates the position of the restriction and cleavage sites. The linearised vector is used in the cloning reaction. (www.lifetechnologies.com). 81

Figure 3.5 Allele frequency for each of ten microsatellite loci with the distribution of alleles indicated for populations of both *T. cordata* and *T. platyphyllos*. 92

Figure 4.1 *T. cordata* samples with newly emerged leaves. 111

Figure 4.2 Location of woods and woodland areas in England where *Tilia* spp. samples were collected (Ordnance Survey, 2014a). 111

Figure 4.3 For the data set of 443 trees the probability of identity, PI and PIsibs for increasing numbers of combined loci. The inset graph has an expanded section of the main graph with values of PI below 0.005 and arbitrary estimate of PI = 1/443 below which trees are unlikely to be related by chance. 126

Figure 4.4 NewHybrids assignment of complete data set of all trees to the population classes: pure *T. cordata*, pure *T. platyphyllos* or to hybrid classes F1 hybrid, F2 hybrid, F1 backcrossed to *T. cordata* or F1 backcrossed to *T. platyphyllos* by posterior probability of class membership. Each bar represents one individual tree and the trees are arranged in their woods (continued). 129

Figure 4.5 *T. cordata, T. platyphyllos,* F1 and F2 tree assignment to population group by wood. The trees that are assigned by percentage with less confidence are also indicated. The 17 woods from the Forest of Bere are grouped as one area having a total population size of 63 trees, an average of 3.7 trees per wood. 132

Figure 4.6 The correlation between latitude and genetic diversity (expected heterozygosity He) of the *T. cordata* populations sampled across England. (This correlation is not significant (P = 0.057)). 138

Figure 4.7 Principal coordinate analysis of pairwise F_{ST} between *T. cordata* populations compared with pairwise F_{ST} of the same populations including the *T. platyphyllos* and hybrids found within those woods. A.) 20 wood populations with putative *T. cordata*. The first two axes explain 32.1% and 19.4% of the total variation. B.) 21 wood populations with *T. cordata*, *T. platyphyllos* and hybrid trees. The first two axes explain 41.8% and 19.9% of the total variation. 141

Figure 4.8 Mantel test for the correlation between geographic distance between 20 *T. cordata* wood populations and genetic distance measured by linearised F_{ST}
A.) *T. cordata*
B.) All *Tilia* (Riseholme and Wenlock Edge *T. platyphyllos* not included) 143

Figure 4.9 Mantel test for the correlation between geographic distance between the Lincolnshire Limewoods *T. cordata* wood populations and genetic distance measured by linearised F_{ST} A.) *T. cordata* B.) All *Tilia* 144

Figure 4.10 FCA plots indicating genetic variance between all the *Tilia* trees sampled. A.) 2D FCA plot for all *Tilia* spp. analysed. *T. cordata* (N = 397) has the least differentiated genetic profile with all trees locating near the origin of the plot. *T. platyphyllos* (N = 19) is the most diverse and hybrid trees (N = 33) locate in the space between the two groups. B.) 2D plot of *T. cordata* (N = 397) with individual trees identified by their wood membership. 146

Figure 4.11 Comparison of estimates for the maximum number of population clusters among all the populations sampled. 147

Figure 4.12 Individual tree assignment to the population structure of *Tilia* spp. derived using STRUCTURE. Plots for K=2 to 6 are shown with individual trees in a.) wood groups and b.) sorted by Q, the estimated membership coefficients of individuals to each cluster. Each cluster K is identified by colour. For values of K=7 and K=8 no addition population structure was observed. (The wood names key is included on the last page of this figure) 149

Figure 4.13 The breakdown of population group A containing 398 putative *T. cordata* trees using STRUCTURE. The population assignment estimates for both K = 2 and K=3 is compared. The key to the wood names is as in figure 4.14. 154

Figure 4.14 The breakdown of population group B (54 trees) using STRUCTURE at the K = 2 level. The population was broken down into 3 population clusters. Group D (34 trees) comprising of all the trees identified as F1 and F2 hybrids by NewHybrids and group C which could be further divided into group E (15 trees) and F (5 trees) containing only *T. platyphyllos*. 155

Figure 4.15 Comparison of estimates for the maximum number of population clusters for putative *T. cordata* data set (group A). a) Evanno *et al* (2005) using ΔK b) Pritchard *et al* (2000) using the maximum value of Ln Pr (X | K). Both methods give the most likely maximum estimate of population clusters as three. 156

Figure 4.16 The Assignment of wood groups to population classes by average posterior probability to each population cluster. The assignment to either K =2 or K = 3 population clusters is compared for all the woods, with the two large areas of woods, the Bardney Limewoods and the Forest of Bere, shown as single populations. The size of the pie charts is related to the number of trees analysed in each area (with a minimum size for small population areas). The number shown after the wood group name is the size of the population analysed in each group 157

Figure 4.17 The assignment of woods of the Lincolnshire Limewoods and the Forest of Bere to population classes by average posterior probability to each for three population clusters. The size of each pie chart is related to the number of trees analysed in each area (with a minimum size for small population areas). The number shown after the wood group name is the size of the population analysed in each group. 158

Figure 4.18 Fine scale spatial genetic structure of trees from within the Chambers Farm Wood group with a distance class size of 50m. This group includes Great Scrubbs, Little Scrubbs, Hatton, Minting and Ivy woods. The autocorrelation coefficient r as a function of the distance between trees is bounded by the upper (U) and lower (L) 95% confidence interval about the null hypothesis of there being a random distribution of trees. The 95% error bars have been derived by bootstrapping over 10^4 replications: - A.) *T. cordata* (N = 68) B.) all *Tilia* spp. (N =85) 160

Figure 5.1 Potential sources of explant tissues (Smith, 2000). I) plant stem, II) flower, III) monocotyledonous seed, IV) dicotyledonous seed. 191

Figure 5.2 Cultures after 34 days. 209

Figure 5.3 The development of explants growing on MS medium containing plant growth regulators: - 215

Figure 5.4 The response of *T. cordata* explants, after 1month in culture on MS medium, to eight plant growth regulator regimes containing varying amounts of the plant growth regulators BAP and NAA (expressed in mg/l). The percentage of the number of explants producing shoots, roots or callus for each MS media is shown. 216

Figure 5.5 Tissue culture progress for successful explants after 5 months culture. 218

TABLES

Table 1.1 The location and area of the individual woods comprising the Bardney Limewoods Sites of Special Scientific Interest (Natural England, 2012). 7

Table 1.2 Identification characteristics of *T. cordata*, *T. platyphyllos* and their hybrids using leaves, shoots and inflorescences taken from the exposed crowns of trees (Pigott, 1969; Wicksell and Christensen, 1999). 22

Table 2.1 Comparison of the four DNA isolation buffers used for the extraction of DNA from *T. cordata* leaf tissue. 47

Table 2.2 CTAB isolation buffer developed for the extraction of DNA from *T. cordata* leaf tissue. 50

Table 2.3 RAPD primer sequences with number of bands, number of polymorphic bands and size range identified in the analysis of 12 *T. cordata* trees. 60

Table 2.4 Dice distance matrix based on the Dice similarity coefficient (Dice distance =1-Dice similarity coefficient). The values are derived from the combined RAPD scores of four primers used with DNA from 12 trees from two woods in the Bardney Limewoods. The two woods, Great Scrubbs Wood (Ch) and Goslings Corner Wood (G), are located approximately 1.4 km apart. 61

Table 3.1 The location of the five Bardney Limewoods *T. cordata* trees in Great Scrubbs Wood that were used for the construction of the microsatellite library. 74

Table 3.2 Characterisation of 11 microsatellite loci cloned for *T. cordata*. The forward (F) and reverse (R) primers sequences are shown together with their observed allele size range, repeat motive and anneal temperature (Ta). 87

Table 3.3 General genetic diversity estimates for two *T. cordata* populations (Chambers Farm Wood, Lincolnshire and Forest of Bere, Hampshire) from the analysis of 11 microsatellites. 89

Table 3.4 General genetic diversity estimates for two *T. platyphyllos* populations (a planted avenue in Riseholme Park, Lincolnshire and a natural population from Wenlock Edge, Shropshire) from the analysis of 11 microsatellites. 89

Table 4.1 Location of the woods from which *Tilia cordata* Mill were sampled, using decimal latitude and longitude coordinates. The wood group and the regional identity (R) of the woods are also indicated. 112

Table 4.2 Genotype input data for NewHybrids with the expected genotype frequency of the hybrid classes from the association between the two populations *T. cordata* (1) and *T. platyphyllos* (0). 116

Table 4.3. The identification of trees with identical allelic structure and trees with alleles that differ by one SSR at one allele. 125

Table 4.4 Genetic diversity characteristics, derived from ten microsatellite markers, of both 20 populations and four regions. *T. cordata* populations are compared with the same populations with all *Tilia* trees sampled (*T. cordata, T. platyphyllos* and hybrids). Also included is a combined group containing trees collected as *T. platyphyllos* from Wenlock Edge and Riseholme Park (planted trees). The mean value over all loci is are given for Na, Ne, Np, Ho, He and F as well as a total mean over all populations and loci. 136

Table 4.5 Summary of the AMOVA analysis for 20 *Tilia* populations. The two population groups analysed were: - A only *T. cordata* trees in the populations B All *Tilia* but without *T. platyphyllos* from Riseholme and Wenlock Edge 139

Table 5.1 Initial development of sterilisation protocols. 200

Table 5.2 Method development plan for tissue culture explant sterilisation. 202

Table 5.3 Plant growth regulator combinations used to stimulate root, callus or shoot development in *T. cordata* tissue culture. 204

Table 5.4 Summary of results comparing explant source for all sterilisation methods. All plants sterilised in 20% bleach (Domestos®) solution and cultured onto MS medium with no plant growth regulators. 206

Table 5.5 The effects of concentration and time of sterilisation on the percentage of uncontaminated explants observed after 34 days of culture. Also shown is the percentage of undamaged explants suitable for reculture after 34 days. 208

Table 5.6 The effect of the inclusion of detergent in the sterilising solution on the percentage of uncontaminated explants and explants suitable for reculture observed after 34 days of culture. 211

Table 5.7 The effect of rinsing the explants after sterilisation on the percentage of uncontaminated explants and explants suitable for reculture observed after 34 days. 213

Table 5.8 The effect of plant growth regulators on the growth of tissue culture samples after 5months in culture (initial sample number of trees = 72, number of tissue samples = 597). 217

Glossary of Abbreviations

AMOVA	Analysis of molecular variance
amp	Ampicillin
ASNW	Ancient Semi Natural Woodland
BAP	6-benzylaminopurine
BP	Years before present
bp	Number of base pairs
BPA	N-benzyl-9-(2-tetrahydropyranyl) adenine
BSA	Bovine serum albumin
ºC	Degrees centigrade
CTAB	Cetyl trimethylammonium bromide
DBH	Diameter at breast height
Det	Detergent
df	Degrees of freedom
DI water	Deionised water
DIECA	Diethyldithiocarbamic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose neuleoside triphosphate
ds	Double stranded
EDTA	Ethylenediaminetetraacetic acid
EthOH	Ethanol
EUFORGEN	European Forest Genetic Resources Programme
F	Fixation index (F-statistics)
FCA	Factorial correspondence analysis
FIS	Inbreeding coefficient (F-statistics)
FIT	Overall fixation index (F-statistics)
FST	Fixation index (F-statistics)
ha	Hectare
He	Expected heterozygosity
Ho	Observed heterozygosity
HOCl	Hyperchlorous acid
HWE	Hardy-Weinberg equilibrium
IBD	Isolation by distance
ITS	Internal transcribed spacers
K	Number of cryptic population groups (used with STRUCTURE analysis)
LB	Luria-Bertani
MCMC	Markov chain Monte Carlo simulation
MS	Murashige and Skoog inorganic formulation
N	Number of individual trees in sample
Na	Number of alleles
NAA	α-naphthaleneacetic acid
NaClO	Sodium hypochlorite
NaDCC	Sodium dichloroisocyanurate
NaOAc	Sodium acetate
Ne	Number of effective alleles
NNR	National Nature Reserve

Np	Number of private alleles
P	Probability
PAWS	Plantations on Ancient Woodland Sites
PCA	Principal coordinate analysis
PCR	Polymerase chain reaction
PI	Probability of identity
PIsibs	Probability of identity of siblings
ppm	Parts per million
PPM™	Plant Preservative Mixture™
psi	Pounds per square inch
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
Q	Membership coefficients of individuals to each population cluster (used with STRUCTURE analysis)
R	Region
r	Auto correlation coefficient
R2	Coefficient of determination
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
Rh	Riseholme Park
RNA	Ribonucleic acid
SAP	Shrimp alkaline phosphatase
s.d.	Standard deviation
s.e.	Standard error
SDS	Sodium dodecyl sulphate
SGS	Spatial genetic structure
SOC	Super optimal broth with catabolite repression
SSC	Saline sodium citrate buffer
SSR	Simple sequence repeats
SSSI	Site of special scientific interest
t	T-test statistic
Ta	Anneal temperature
TBE	Tris borate EDTA buffer
TE	Tris EDTA buffer
TLE	Tris low EDTA Buffer
Tris	Tris(hydroxymethyl)aminomethane
Tris-Cl	Tris(hydroxymethyl)aminomethane hydrochloride
UPGMA	Unweighted pair group method with arithmetic averages
UV	Ultra violet
VNTR	Variable number tandem repeat
WE	Wenlock Edge
WPM	Woody plant medium
2,4D	2,4-dichlorophenoxyacetic acid
2D	Two dimension
3D	Three dimension

CHAPTER ONE

General Introduction

1.1 Introduction

The Lincolnshire Limewoods are a group of Ancient Semi Natural Woodlands within Central Lincolnshire that include the nationally important Bardney Limewoods National Nature Reserve. These woods are an important reservoir for biodiversity in the region and are managed to improve and increase the natural habitats in the area (Lincolnshire Biodiversity Partnership, 2011). The Limewoods, which contain significant populations of *Tilia cordata* Mill., are highly fragmented and are isolated from each other by farmland. Isolated populations are potentially at risk of experiencing a reduction in genetic diversity as a consequence of increased levels of inbreeding and genetic drift (Couvet, 2002). In plants, however, lifestyle factors such as range, fecundity, method of reproduction and generation time have also been found to affect the genetic diversity (Hamrick *et al*., 1979; Loveless and Hamrick, 1984). Consequently, large, long-living tree species that have extensive continuous populations, outcrossing breeding systems and long distance pollen and seed dispersal, generally maintain high levels of genetic diversity (Hamrick *et al*., 1992).

The conservation of *T. cordata* is of interest throughout Europe. The European Forest Genetic Resources Programme (EUFORGEN) (EUFORGEN, 2011), a collaboration of European countries promoting conservation and sustainable use of forest genetic resources, includes *T. cordata* in its Noble Hardwood Network and has produced a technical guideline for "the genetic conservation and use of *Tilia* spp." (Svejgaard Jensen, 2003). Conserving woodlands and their native tree species results in increased biodiversity by creating and sustaining unique habitats and protects the environment by acting as a carbon store, increasing soil fertility and storing water (Aerts and Honnay, 2011).

1.2 The Bardney Limewoods

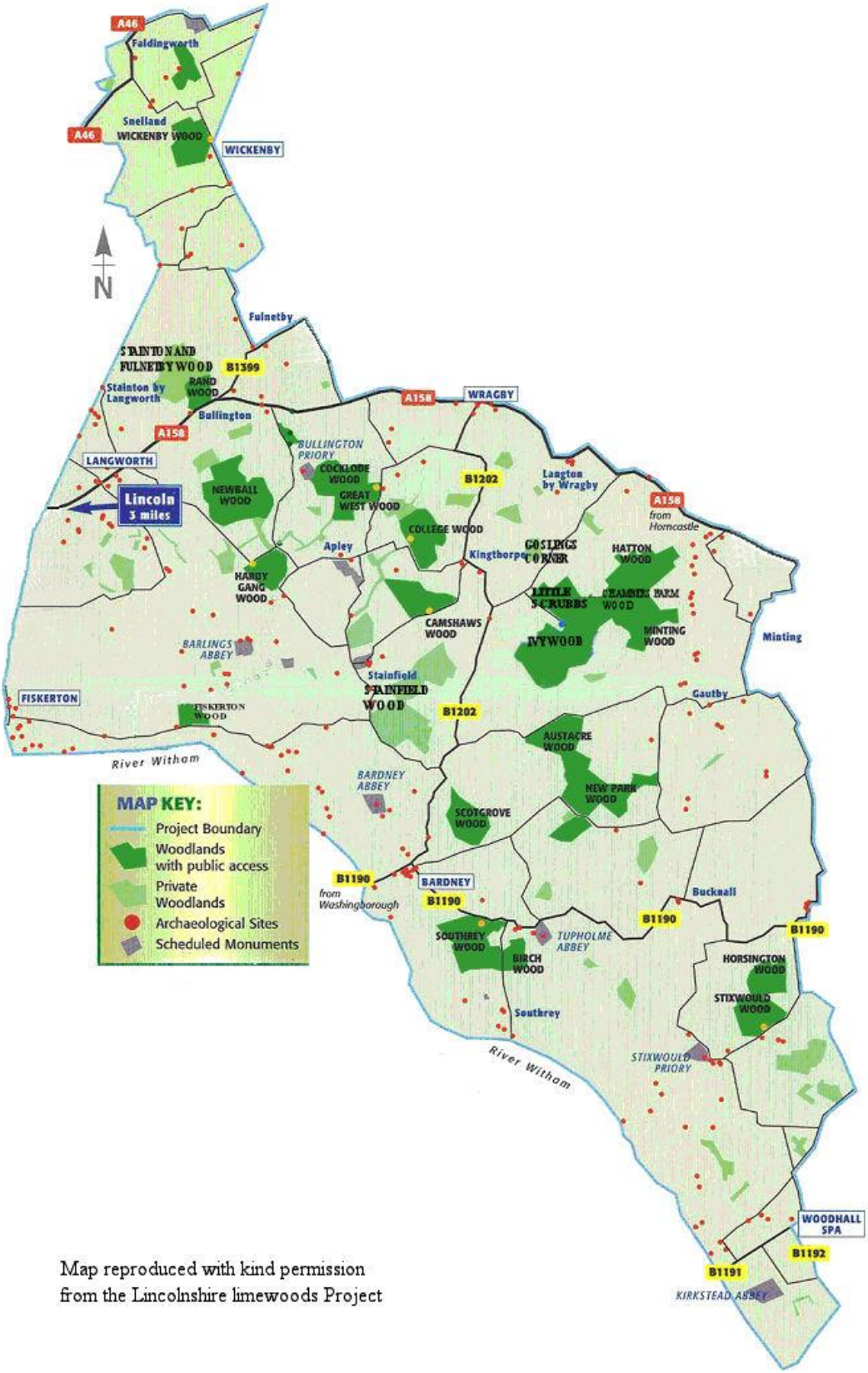
The greatest concentration of limewoods in Britain occurs in the central clay vale of Lincolnshire (Ratcliffe, 2012). This area, containing many fragmented Ancient and Semi-Natural Woodlands (ASNW), is known as the Lincolnshire Limewoods (figure 1.1). The importance of these ASNW has been recognised by botanists for many years and a number of the largest, most species-rich woods, which have also been less affected by recent management changes, have been designated as Sites of Special Scientific Interest (SSSI) since the 1980s. These nine woods are now grouped together as the Bardney Limewoods SSSI (table 1.1). In 1997 the SSSI woods within the Bardney Limewoods which were owned by the Forest Enterprise (now the Forestry Commission), were declared a National Nature Reserve under section 19 of the National Parks and Access to the Countryside Act 1959 and Section 35 of the Wildlife and Countryside Act 1981. These woods have a total area of 383.6 ha. However, there are many other woods in this area containing important populations of *T. cordata*, including Goslings Corner SSSI, which is a Lincolnshire Wildlife Trust Reserve and one of the last remaining relics of the once very extensive Langton Wood.

All of these woodlands, together with a number of other sites of historical and archaeological interest, were included in the Lincolnshire Limewoods Project (Lincolnshire Limewoods Project, 2007) which covered an area of 155 km^2 from Wickenby in the north to Woodhall Spa in the south of the county (figure. 1.1). This project, supported by significant heritage lottery funding, facilitated the planting of 130 hectares of new woodlands and over 26km of new hedgerows to restore linkages between important woodland habitats.

The survival of the Bardney Limewoods over the centuries is possibly a consequence of the location of the woods and their situation on neutral or acid boulder clay, sometimes overlain with sands and gravels (Gibbons, 1975). The remote location of many of the woods, often on the edge of parish boundaries, may have made small-scale timber extraction unprofitable. Conversion to arable farmland may also have been impractical on land that is poorly drained and difficult to cultivate mechanically. In the twentieth century the survival of many ASNW, including the Bardney Limewoods, was threatened by changing forestry management practices. Prior to the Second World War the Bardney Limewoods had been managed using traditional forestry practices. Trees such as oak, together with Scots pine and Norway spruce, were planted and former agricultural land between the ancient woodlands at Chambers Farm was planted with traditional tree mixtures (Haggett, 1997). By the end of the Second World War, although most of the native broadleaved trees had been felled, with no other management taking place, coppiced lime, oak, ash, field maple and birch were able to regenerate into high forest. After the war, however, government policy, as expressed by the objectives of the 1951 Forestry Act, promoted the establishment of 2,000,000 ha of productive woodlands (Nail, 2008). As a consequence, the Bardney Limewoods, like many other native broadleaf woodlands in Britain (Rackham, 2008), were cleared and treated with herbicides with the aim of eradicating the broadleaf species to enable their conversion to conifer plantation (Haggett, 1997). Fortunately this strategy was not successful and the native trees survived. More sympathetic management of the woods after 1971 led to the eventual formation of the Forest Nature Reserve in 1989 and the Bardney Limewoods National Nature Reserve in 1997.

In Ancient and Semi-Natural Woodland (ASNW) which is owned by the Forestry Commission and planted with conifers, the policy is now to remove these non-native species as part of the Forestry Commission's remit to restore native woodland on ancient woodland sites. Since the declaration of the National Nature Reserve, the Forestry Commission has undertaken to manage all of their Lincolnshire Limewoods, including those not designated SSSI, to increase the habitat for animals and insects as well as ancient woodland plant species. To facilitate this, some of the woods are being returned to traditional coppice, with others, such as Hatton Wood, managed as high forest or as 'research natural' areas, which will have minimal intervention. A network of rides in each wood is also managed to provide wide grassed verges and open 'glade' areas within the wood with the aim of improving biodiversity. It is also intended that, as recent conifer plantations on grassland and farmland reach maturity and are felled, these sites will be allowed to revert to native woodland by natural regeneration.

Figure 1.1 Map of the Lincolnshire Limewoods Project Area. This area includes the nine SSSI woods that comprise the Bardney Limewoods National Nature Reserves.



Map reproduced with kind permission from the Lincolnshire limewoods Project

Table 1.1 The location and area of the individual woods comprising the Bardney Limewoods Sites of Special Scientific Interest (Natural England, 2012).

SSSI woods which are part of the Bardney Limewoods	Latitude	Longitude	Area (Ha)
College Wood	53.27606	-0.34617	49.77
Great West and Cocklode Wood	53.27606	-0.34617	51.20
Hardy Gang Wood	53.25913	-0.35774	26.87
Hatton Wood*	53.25993	-0.25726	36.93
Little Scrubbs Wood *	53.25407	-0.28808	13.80
Ivy Wood *	53.24873	-0.28543	15.21
Newball Wood	53.27048	-0.36716	57.59
Scotgrove Wood	53.21993	-0.30792	29.20
Wickenby Wood	53.33368	-0.38096	43.56

* Part of the Chambers Farm Wood group

1.3 Ancient and Semi-Natural Woodland

All of the limewoods in the Bardney Limewoods Nature Reserve and a number of other non-SSSI limewoods within the Lincolnshire Limewoods are classed as Ancient and Semi-Natural Woodland (ASNW). Ancient woodlands are defined as having been in existence for more than 400 years, i.e. from before 1600 when widespread tree planting and record keeping began. They are also classed as semi-natural because, although they have been managed as a resource for the local community over many centuries, they have always existed as woodland (Peterken, 1996). The origin of the Lincolnshire Limewoods, however, is likely to be significantly earlier than this.

In the 1970's George Peterken (1974) developed a method of assessing the age of woodland based on the vascular plants found there. As a result of this, English Nature extended this research and collated a table of ancient woodland indicator plants (AWI) for the regions of England (Goldberg and Kirby, 2002/3). The Lincolnshire Limewoods are rich in both variety and number of ancient woodland indicator plants (figure 1.2); this, together with records from old maps and the Domesday Book, indicates that these woods are most likely of ancient origin.

The value of ancient woodlands is considerable. For local communities many woods are important public spaces that have historical and archaeological significance. The stable conditions that exist in the ancient woodlands encourage significant biodiversity with distinctive flora and fauna and can provide a habitat for rare and threatened species. Maintaining woodlands can help preserve physical features such as natural streams and soil profiles as well as providing a reservoir of genetic diversity (Peterken, 1996).

Figure 1.2 *T. cordata* together with *Hyacinthoides non-scripta* (L.) Rothm. and *Anemone nemorosa* L.; some of Lincolnshire's ancient woodland indicator plants (Scotgrove Wood spring 2011).

1.4 The Origin of Ancient Limewoods in Britain

At the end of the last glacial period, the Devensian, 12,000 to 11,500 years before present (BP), pollen records indicate that, as the ice sheet receded, the flowering vegetation in Britain was similar to that covering the tundra today. Analysis of pollen and plant fossils suggest that grasses, sedges and herbaceous plants predominated, together with dwarf shrubs such as crowberry (*Empetrum nigrum*) and dwarf trees such as *Betula* (*Betula nana* L.), *Juniperius* (*Juniperus communis L.*) and *Salix* (*Salix herbacea* L.) (Conolly *et al*., 1950; Atherden, 1992; Rhind and Jones, 2003). At this time, with water held within

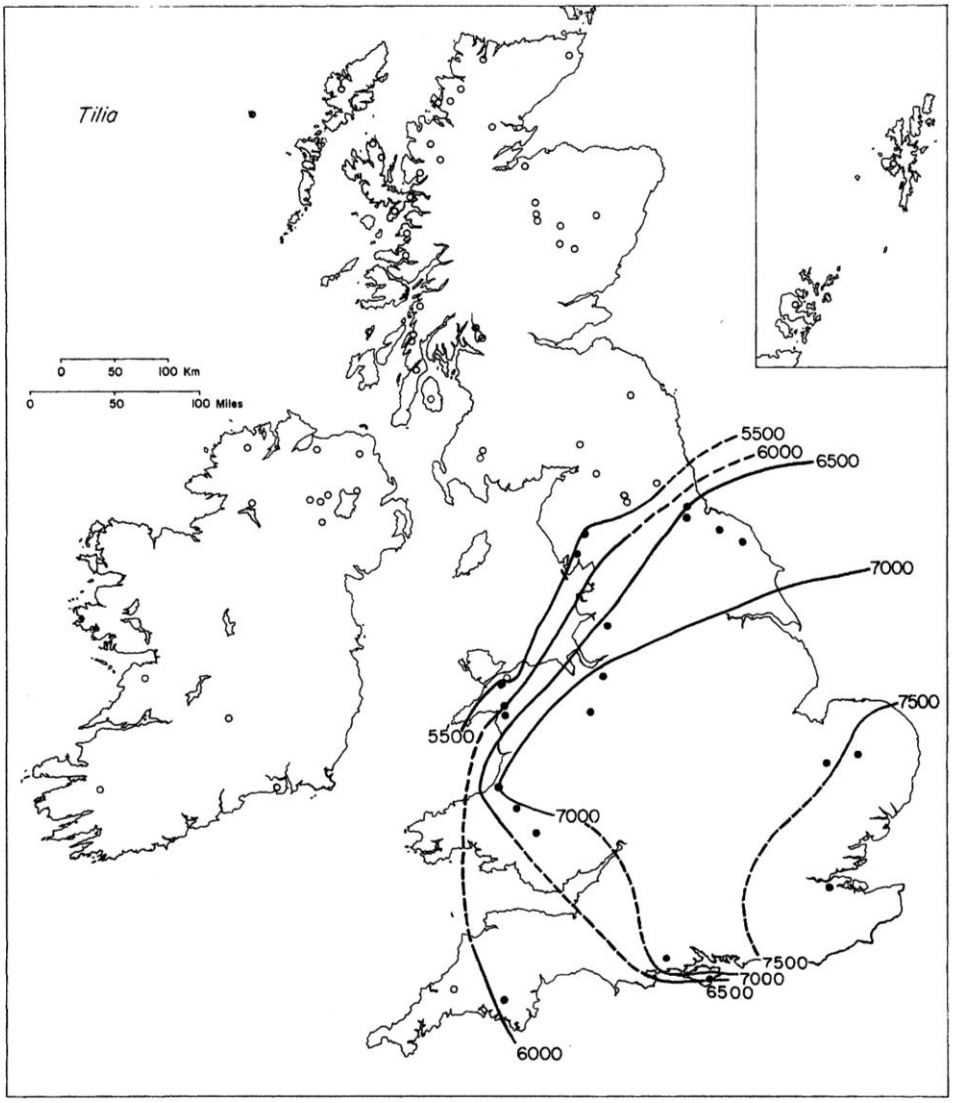
the retreating ice sheets, the sea levels were low and Britain was connected to the European mainland with low lying land to the south and the east (Fitch *et al*., 2005). At the time of the last glacial maximum (27,000 to 21,000 BP) Fairbanks (1989) estimated the sea level to be 120 m below its present level. With rising temperatures (Lang *et al*., 2010) plants and animals were able to spread from glacial refugia in Europe (Provan and Bennett, 2008) to re-colonise the British landscape. Pollen records (Birks, 1989) indicate that the tundra vegetation was initially replaced by arctic tree species such as birch, aspen and sallow. As the climate warmed further, birch trees were replaced, first by pine and hazel, then by oak and alder and then by lime and elm, these being more suited to the increasing temperatures (Godwin, 1940; Bennett, 1988; Rackham, 2010). Rising sea levels, as a consequence of the melting ice sheets, eventually divided Britain from Europe and around 8,000 BP Britain became an island (Gupta *et al*., 2007; Weninger *et al*., 2008; Sturt *et al*., 2013).

T. cordata and *T. platyphyllos* arrived in Britain after the end of the last ice age, when temperatures rose (Marcott *et al*., 2013) and climate conditions became suitable for these thermophilous trees. *Tilia*, after arriving in Britain some time before 7,500 BP, spread to its most northerly limit by 5,500 BP (figure 1.3 (Birks, 1989)). At this time it had become the dominant tree species in the south east of England (Birks *et al*., 1975). The records show that, around 5,000 BP, elm pollen declined rapidly all over Western Europe and Britain, possibly due to disease (Smith and Pilcher, 1973; Rackham, 1980). Declines in small-leaved lime pollen around 3,000 BP (Grant *et al*., 2011) were also noted and Turner (1962) suggests that this decline is closely associated with human activity in the Neolithic and Bronze Ages. By the time of the Roman invasion in 2,000 BP, much of the ancient

woodlands had been cleared and pollen records show a reduction in tree pollen in general and increases in cultivated plant and grass pollen (Dumayne, 1993).

Rackham (2001) indicates that records from the Domesday Book show that by 1086 AD woodland was not extensive in England. In Lincolnshire only 4% was wooded, with 432 woods recorded as either wood pasture or coppice. The limewoods were, however, an important resource for the local community and would have been used to provide bast for the manufacture of rope, poles for building, fencing and charcoal, fodder for animals, wood for carving and honey. By 1895, however, increasing demand for agricultural land had reduced this area and in Lincolnshire only 3% remained wooded (Board of Agriculture returns, Rackham, 2001). In the twentieth century many ancient woodlands were replanted with conifers and the dense shade in the conifer plantations altered their biological diversity (Rackham, 2001). Today, although 4% of Lincolnshire is wooded, only 1% can be classed as Ancient Semi-Natural Woodland (ASNW) (Lincolnshire Biodiversity Partnership, 2011).

Figure 1.3 Isochrone map of the increasing extent of *Tilia* pollen in the British Isles over time. The isochrones estimate the date (years BP) of the palynological evidence of the presence of *Tilia* pollen (Birks, 1989).



1.5 The Characteristics of *Tilia cordata* Miller

Tilia cordata Mill. and *Tilia platyphyllos* Scop. are members of the Malvaceae family in the order of Malvales and are in a group of four *Tilia* species that are native to Europe (Pigott, 2012). *T. cordata* and *T. platyphyllos* are distributed throughout Europe, with *T. cordata* being more numerous and widespread and found at more northerly latitudes than *T. platyphyllos* (Svejgaard Jensen, 2003) (figure 1.4).

T. cordata can grow into a large tree and standards within woodlands may reach up to 30m (Pigott, 1991) (figure 1.5). More usually, however, the trees are managed within the woods and are found as coppiced stools with many stems. Coppicing is usually on a 15- or 25-year cycle (figure 1.6). If the coppice is left unmanaged several dominant large trunks can arise (figure 1.7). This ability to regenerate from shoots emerging from basal sprouts and epicormic buds within the bark, or from natural layers occurring where branches touch the ground, ensures the survival of *T. cordata* over many centuries (Pigott, 1991). The trees have smooth, dark grey bark when young but develop deep longitudinal fissures with age. Young shoots usually have red bark with buds that are arranged as a spiral around the stem (figure 1.8).

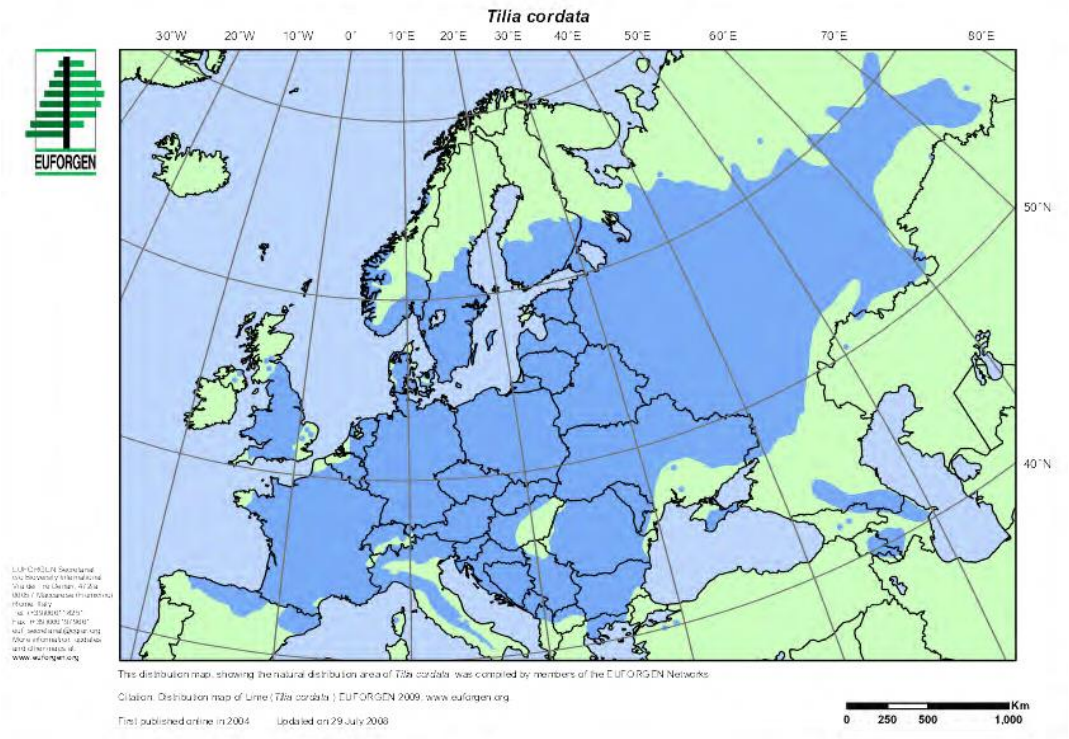
The leaves are cordate (heart-shaped) with a dendrate margin. The underside of the leaf has small tufts of hair in the vein axis. Where the leaves are in sunlight, the lower surface is glaucous and the tufts of hair brown (figure 1.9). Leaves that are in the shade are less robust, green on both sides and the hairs paler or white. Leaves that occur on basal shoots, e.g. after coppicing, are usually larger than those found on the mature trees.

The trees flower between late June and early August, depending on latitude, and are pollinated by insects (Pigott and Huntley, 1981). The flowers usually occur high in the tree canopy in full sunlight. They are hermaphrodite and protandrous, with the anthers maturing before the stigmas. The inflorescences of *T. cordata*, with between 5 and 11 pale yellow flowers with 5 petals, are attached to a leafy bract and are held erect above the leaves (figure 1.10). In *T. platyphyllos* and *Tilia* hybrids, however, the inflorescences are pendulous, hanging below the leaves; this appears to be a dominant trait (Pigott, 1991) (figure 1.11).

The fruit, usually containing one seed, is round or ellipsoidal (3 to 4 mm diameter) and covered with dense, brown fasciculate hairs. It is unlike that of other *Tilia* species in that the seed case is without ribs. Fertile seeds often remain on the trees until December (Pigott, 1991). The seeds require a period of vernalisation before germination can occur and, even then, germination may not occur for 18 months or more (Pigott, 1991). Seedlings are uncommon in the Lincolnshire Limewoods but figure 1.12 shows *T. cordata* seedlings from Hatton Wood, observed in May 2012 when many seedlings were found. These seedlings, however, were not evident on return to site in August 2012.

T. cordata is entomophilous and relies on many different insects to facilitate pollination (Pigott, 1969; Pigott and Huntley, 1981). Anderson (1976), however, found that wind pollination was also possible. Even though mechanisms, such as the flowers being protandrous, exist to minimise the possibility of self-fertilisation, investigations by Pigott and Huntley (1981) indicate that *T. cordata* is not generally self sterile (although some trees were found to be so).

Figure 1.4 The European distribution of *Tilia cordata* Mill (EUFORGEN, 2008a) and *Tilia platyphyllos* Scop.(EUFORGEN, 2008b).



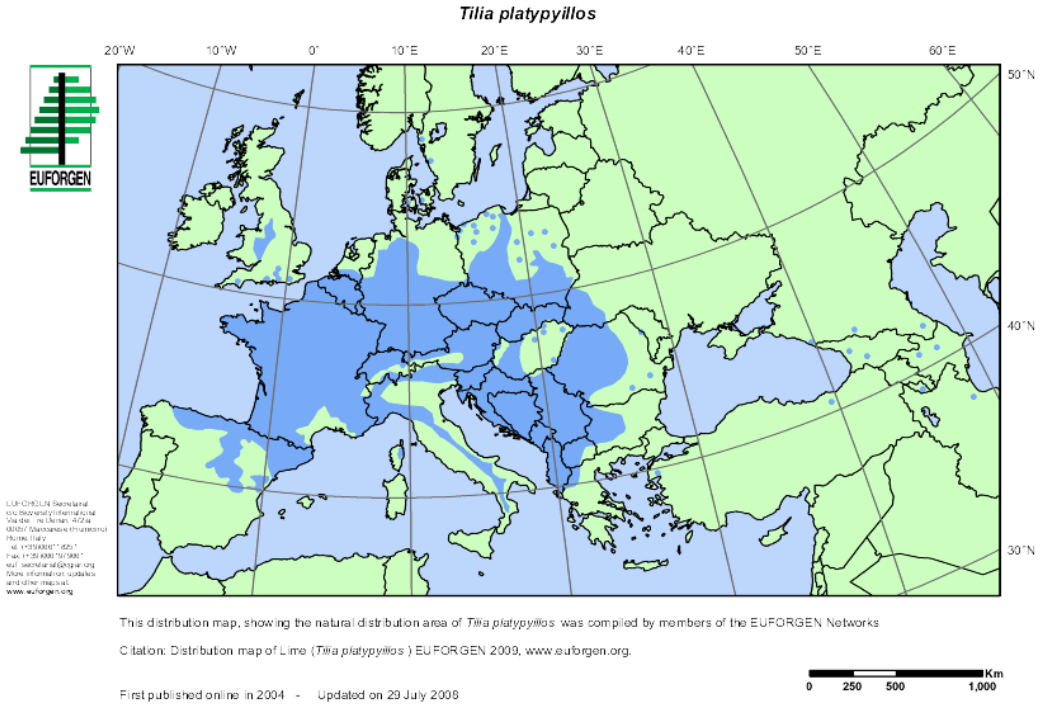


Figure 1.5 Mature standard *T. cordata* in Potterhanworth Wood, Lincolnshire.

Figure 1.6 *T. cordata* coppiced stool less than 5 years old.

Figure 1.7 Four dominant trunks of a mature *T. cordata* coppiced tree.

Figure 1.8 Young red barked stems of *T. cordata* before leaves emerge.

Figure 1.9 A comparison between the abaxial leaf surfaces of *T. cordata* and *T. platyphyllos*.

a.) *T. cordata*: glaucous underside of leaf and orange brown tufts of fasciculate hairs in the leaf axils.
b.) *T. platyphyllos*: white simple hairs along on the main leaf vein.
c.) Abaxial surface of a whole leaf of *T. cordata* (left) and *T. platyphyllos* (right).
d.) Leaf of *T. cordata* growing in the shade with small tufts of white hair in the vein axils.

a.)

b.)

c.)

d.)

Figure 1.10 *T. cordata* flowers held erect above the bracts above the leaf canopy.

Figure 1.11 Comparison between the erect flower bracts of *T. cordata* (left) and the pendulous bracts of *T. platyphyllos* (right).

Figure 1.12 *T. cordata* seedlings from Hatton Wood, Lincolnshire in May 2012.

Two native *Tilia spp*. are found in the British Isles; the small-leaved lime, *Tilia cordata* Mill. and the large-leaved lime *Tilia platyphyllos* Scop., with *T. cordata* being the more common and widely distributed (figure 1.4 (EUFORGEN, 2008a; EUFORGEN, 2008b)). The morphology and the history of the trees have been described by Pigott (1991; 2012). Where the two species grow together natural hybrids are occasionally produced (Pigott, 1991), even though *T. platyphyllos* flowers 10 to14 days earlier than *T. cordata*. In the absence of inflorescences, leaf morphology has been used in several studies to identify the incidence of hybrids and *T. platyphyllos* within *T. cordata* populations (Logan *et al*., 2015; Phuekvilai, 2014; Pigott, 1969; Wicksell and Christensen, 1999). The morphological features used to differentiate between the two species and their hybrids are shown in table 1.2 (Pigott, 1969; Wicksell and Christensen, 1999). Pigott (1969) describes ancient semi-natural woods in Derbyshire where *T. cordata*, *T. platyphyllos* and hybrids occur together and Logan *et al* (2015) investigated the occurrence of hybrids within woods in Derbyshire, South Yorkshire, Worcestershire, Shropshire and Gloucestershire. Specimens of *T. platyphyllos* and the hybrid *T. x europaea*, often introduced from Europe, have been widely planted in parks and towns and were popular trees in the seventeenth and eighteenth centuries (Pigott, 1992).

The hybrid trees are sometimes fertile and have physical features that are either similar to one of the parent species or intermediate to them both. Investigation of the pollen of hybrids shows that, in some cases, the exine patterns of *cordata* and *platyphyllos* occur on opposing sides of the pollen grain (Andrew, 1971). The frequency of hybridisation is, however, low and Fromm and Hattemer (2003) report in their study using allozymes that the occurrence of interspecific hybridisation and introgression is restricted. Pigott (1969)

also suggests that the amount of variation between the trees found in Derbyshire indicates only a limited occurrence of introgessive hybridisation.

Table 1.2 Identification characteristics of *T. cordata*, *T. platyphyllos* and their hybrids using leaves, shoots and inflorescences taken from the exposed crowns of trees (Pigott, 1969; Wicksell and Christensen, 1999).

T. cordata	*T. cordata* x *T. platyphyllos* hybrids	*T. platyphyllos*
Leaves		
Largest leaves on second order shoots < 8cm long	Largest leaves on second order shoots 8 - 10cm long	Largest leaves on second order shoots > 10cm long
Adaxial surface flat; tertiary veins not raised on abaxial surface	Intermediate	Adaxial surface rugose; tertiary veins prominent on abaxial surface
Abaxial surface glaucous	Intermediate	Abaxial surface green
Brown hairs in axils of veins on abaxial surface	Pale hairs in axils of veins on abaxial surface	No tufts of hairs in axils of veins on abaxial surface
Veins hairless on abaxial surface	Veins with scattered hairs on abaxial surface	Veins very hairy on abaxial surface
No hairs between veins on abaxial surface	Scattered hairs between veins on abaxial surface	Hairy between veins on abaxial surface
No hairs on adaxial surface	Scattered hairs on adaxial surface	Many hairs on adaxial surface
Petiole		
<1.2 mm diameter	1.2 – 1.5mm diameter, few hairs	> 1.5mm diameter
No hairs	Few hairs	Many hairs
Young twigs		
No hairs	Few hairs	Many hairs
Inflorescence		
Obliquely erect	Pendulous	Pendulous

1.6 Regeneration of *Tilia cordata* within the Limewoods

Regeneration of *T. cordata* occurs in several ways, either sexually, through the production of seeds, or vegetatively by layering, cuttings or by coppicing. In England, however, natural regeneration of native *T. cordata* has been infrequent and rejuvenation of the trees is primarily by coppicing and natural layering. Although *T. cordata* may begin to produce flowers at between 10 and 20 years old, trees that are within the wood itself will not flower if shaded and, therefore, only flower when the tree reaches the top of the forest canopy. Managing the woods by coppicing, with a typical 15-year cycle, means that, as the trees fail to grow to maturity, they may never produce seeds. Trees from central Europe produce fertile seeds in most years but, in England, fertile seeds tend only to occur in years when July and August are exceptionally warm (Pigott, 1991). Pigott and Huntley (1981) found that low temperatures at the time of flowering inhibit fertilisation for *T. cordata* and low temperatures as the seeds ripen can also prevent the embryos from developing. Even in years when fertile seeds are produced, seedlings are rarely found (Pigott, 1991). Seeds and seedlings that are produced are liable to be destroyed by bank voles (*Clethrionomys glareolus* Schreber) and wood mice (*Apodemus silvaticus* L.) within the first three years of growth (Pigott, 1985); roe deer (*Capreolus capreolus* L.) are also known to cause serious damage to young growth (Pigott, 1991). These factors, which have been observed to affect the lime trees in woods in Lancashire, Surrey (Pigott, 1985) and Derbyshire (Pigott, 1969), are probably also significant in the lack of successful sexual reproduction of *T. cordata* in the Lincolnshire Limewoods.

The trees within the Limewoods have maintained their position within the ecosystem because of the ‘very great longevity of individual trees and their extraordinary potential for vegetative regrowth after injury’ (Pigott and Huntley, 1978). When stems of *Tilia*

collapse with old age or are removed by coppicing the trees respond by producing new growth, which has the potential to form a mature tree. This process enables the trees to survive for many centuries. Pigott (1989) has estimated that some of the trees surveyed in the northwest are over 200 years old, with the larger coppiced stools between 1,300 and 1,900 years old. Regeneration of *T. cordata* can also occur by layering. Layers made around coppiced stools at Chambers Farm Wood in 2007 have produced roots and shoots that will possibly produce new trees which are clones of the parent (figure 1.13). Rooting and new growth can also occur naturally where either branches bend downwards and touch the ground (figure 1.14) or trees fall without breaking all their roots (figure 1.15).

With the aim of increasing biodiversity, the Lincolnshire Biodiversity Action Plan (Lincolnshire Biodiversity Partnership, 2011) for the Lincolnshire Limewoods promotes the creation of new habitats and connectivity between adjoining woodlands. The possibility of increasing the area of indigenous *T. cordata* within the Lincolnshire Limewoods National Nature Reserve, using natural regeneration from locally sourced seeds, may be constrained, however, as a consequence of the limited number of seedlings and viable seed produced. Coppicing and layering, although successful in regenerating the old trees, do not enable the trees to extend their range in any significant way. It is important that, if *T. cordata* is introduced to newly created woods in Lincolnshire, it reflects both the adaptive and genetic diversity of the trees that are already there (Friar *et al.*, 2001). George Peterken writes in Natural Woodland:

> "widespread and unrecorded re-introduction of, say, lime and service would eventually destroy these species as sources of information about the long-term effects of people on the environment."(Peterken, 1996)

Successful tissue culture of *T. cordata* could also provide a source of planting material for new woodlands in the absence of viable seeds or successful cuttings. Trees such as black alder (*Alnus glutinosa* (L.) Gaertn. (San Jose *et al*., 2013) and *Calophyllum apetalum* Willd. (Nair and Seeni, 2003) have been successfully micropropagated for reintroduction into native habitats. Research is ongoing into the potential of using this technique for endangered tree species such as *Juniperus communis* L. (Kocer *et al*., 2011) and the Chilean tree *Gomortega keule* (Mollina) Baillon (Muñoz-Concha and Davey, 2011). When successful, plants produced using micropropagation techniques may have rejuvenated characteristics and be disease free, making them highly suitable for replanting in restoration projects (Bonga, 1982; Webster and Jones, 1989; Ballester and Vieitez, 2012).

Figure 1.13 Layered *T. cordata* coppiced stool from Great Scrubbs Wood, Lincolnshire.

a.) 2007

b.) 2009

Figure 1.14 Self-layered *T. cordata* branch; from Hardy Gang Wood, Lincolnshire.

Figure 1.15 Fallen *T. cordata* tree still growing and producing vertical shoots that may possibly root; from Skellingthorpe Old Wood, Lincolnshire.

1.7 The study of genetic variation and structure in *Tilia* spp.

Genetic variation and structure is determined by biological, historical and ecological factors that act upon populations. Ecological factors can affect genetic structure by acting upon reproduction and dispersal strategies (Loveless and Hamrick, 1984). As for many temperate plant species, the genetic variations that exists in *T. cordata* and *T. platyphyllos* populations are, in part, a consequence of the recolonisation of Europe at the end of the last glacial period (Hewitt, 1999; Provan and Bennett, 2008). As the climate warmed, rapid northwards expansion through a series of founder events led to northern populations having less genetic diversity and an increase in homozygosity (Hewitt, 1999).

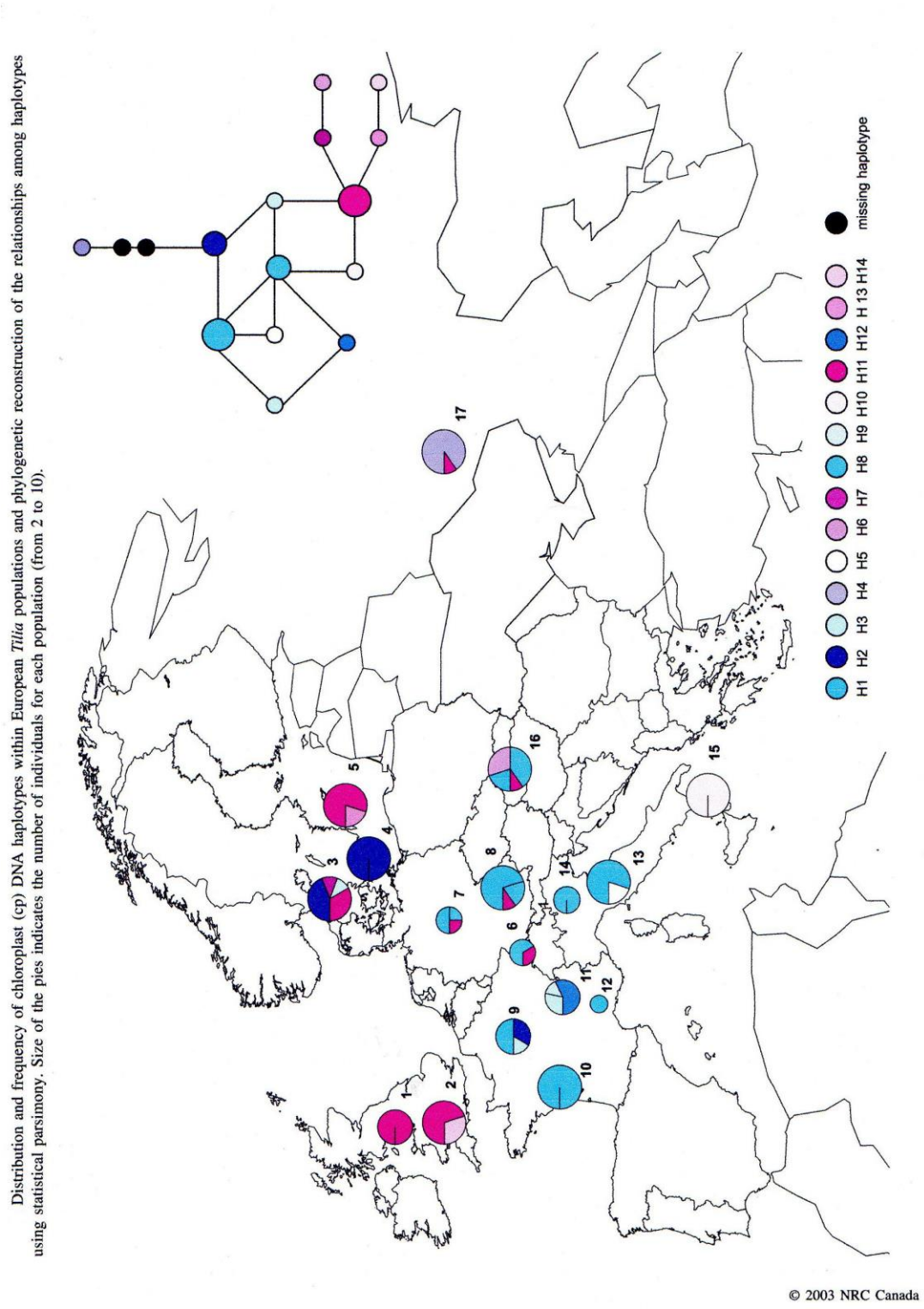
Molecular markers have been used to study various aspects of *Tilia* genetic diversity. Random Amplified Polymorphic DNA (RAPD) was used by Hosseinzadeh Colagar *et al* (2013) to study six closely located populations of *Tilia rubra* D.C. in Iran and identified low inter-population differentiation between the groups. Liesebach and Sinkó (2008) also used RAPD and identified the *Tila* ancestor species of hybrid planted avenue trees. Allozymes were used by Fromm and Hattemar (2003) to investigate the incidence of hybridisation between *T. cordata* and *T. platyphyllos* within mixed woodlands. Internal transcribed spacers (ITS) from nuclear ribosomal DNA were used by Yousefzadeh *et al*. (2012) to identify *Tilia* spp. within the Hyrcanian Forest in Iran.

Phylogeographic studies have been carried out using PCR-RFLP to measure chloroplast diversity between European populations of *T. cordata* (Fineschi *et al*., 2003). The distribution and frequency of the chloroplast haplotypes suggested low genetic differentiation between the populations, although there was some evidence of weak phylogeographic structure, and possible glacial refugia were identified in Western Russia

and central Italy (figure 1.16). More recently, Phuekvilai (2014) has used chloroplast haplotypes and genomic DNA microsatellite analysis to identify genetic structure among European populations and found some *T. cordata* and *T. platyphyllos* chloroplast haplotypes to be shared. The Caucasus, Iberia, Italy and the Balkans were proposed as the locations of refugia for *T. cordata* during the last glacial period, with *T. platyphyllos* possibly surviving in Iberia, Italy and the Balkans. This study also suggests that *T. cordata* may have colonised Britain from refugia in Italy, the Balkans and Iberia, whereas the *T. platyphyllos* postglacial origins lie within the Iberian Peninsula, as do *Quercus petraea* (Matt.) Liebl. and *Quercus robur* L. (Cottrell, 2002).

Microsatellite markers, as a result of their diversity and high mutation rate, are particularly suitable for the investigation of populations in the recent past i.e. 10 to 100 generations (Beaumont and Bruford, 1999; Selkoe and Toonen, 2006). They can also provide insight into ecological questions on a local level but they are less suitable for broad studies across taxonomic groups (Selkoe and Toonen, 2006).

Figure 1.16 Distribution and frequency of chloroplast DNA haploypes within European *Tilia cordata* populations **(**from Fineschi *et al*. (2003)).



1.8 Aims of the Study

The iconic tree of the Lincolnshire Limewoods is *T. cordata*. The trees most likely arrived in Lincolnshire after the end of the last glacial maximum after spreading from glacial refugia in Europe. Over the past centuries the lime trees have been managed by coppicing and the resources obtained used within the local communities and now the trees are an important part of the rich diverse habitat that exists within the woods. The aim of this study was to investigate the genetic diversity and structure of *T. cordata* within the Lincolnshire Limewoods in order to inform suitable conservation strategies for the woods and for newly created woodland habitat.

Chapter 2 Techniques were developed to extract *T. cordata* DNA of suitable quality for use in subsequent Polymerase Chain Reactions. A pilot study with Random Amplified Polymorphic DNA was carried out to test the feasibility of using this molecular marker to identify genetic structure among the trees.

Chapter 3 A microsatellite library was developed for a *T. cordata* genetic investigation and ten microsatellite primers were characterised for *T. cordata* populations. The microsatellites were also found to be transferable to *T. platyphyllos*.

Chapter 4 Microsatellite markers were used to determine the genetic diversity and the genetic structure of the trees within the Lincolnshire Limewoods. These results were compared with those of other *T. cordata* populations from outside Lincolnshire. Clonal groups were detected as well as the occurrence and distribution of *T. platyphyllos* and *T. cordata x T. platyphyllos* hybrids.

Chapter 5 Techniques were developed that might enable planting material for new woods to be sourced from the micropropagation of locally selected trees.

Chapter 6 A general discussion of the overall results of this study.

1.9 References

Aerts, R. and Honnay, O. (2011) Forest restoration, biodiversity and ecosystem functioning. *BMC Ecology*, 11 29.

Anderson, G.J. (1976) The pollination biology of *Tilia*. *American Journal of Botany*, 63(9) 1203-1212.

Andrew, R. (1971) Exine pattern in the pollen of British species of *Tilia*. *New Phytologist*, 70(4) 683-686.

Atherden, M. (1992) *Upland Britain: a natural history*. Manchester University Press.

Ballester, A. and Vieitez, A.M. (2012) Partial rejuvenation of mature hardwood trees through somatic embryogenesis: The example of pedunculate oak. In: Y. S. Park and J. M. Bonga (eds.) *Proceedings of the IUFRO Working Party 2.09.02 Conference on "Integrating Vegetative Propagation, Biotechnologies and Genetic Improvement for Tree Production and Sustainable Forest Management*". Brno, Czech Republic, 25-28 June 2012, 47-55.

Beaumont, M.A. and Bruford, M.W. (1999) Microsatellites in conservation genetics. In: D. B. Golstein and C. Schlotterer (eds.) *Microsatellites Evolution and Applications.* 1st edition. Great Britain: Oxford University Press, 165-182.

Bennett, K.D. (1988) Holocene pollen stratigraphy of central East Anglia, England, and comparison of pollen zones across the British Isles. *New Phytologist*, 109 237-253.

Birks, H.J.B. (1989) Holocene isochrone maps and patterns of tree-spreading in the British Isles. *Journal of Biogeography*, 16(6) 503-540.

Birks, H.J.B., Deacon, J. and Peglar, S. (1975) Pollen maps for the British Isles 5000 years ago. *Proceedings of the Royal Society of London.Series B, Biological Sciences*, 189(1094) 87-105.

Bonga, J.M. (1982) Vegetative propagation in relation to juvenility, maturity and rejuvenation. In: J. M. Bonga and D. J. Durzan (eds.) *Tissue culture in forestry*. Netherlands: Martinus Nijhoff, 387-412.

Conolly, A.P., Godwin, H. and Megaw, E.M. (1950) Studies in the post-glacial history of British vegetation. XI. Late-glacial deposits in Cornwall. *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences*, 234(615) 397-469.

Cottrell, J.E., Munro, R.C., Tabbener, H.E., Gillies, A.C.M., Forrest, G.I., Deans, J.D. and Lowe, A,J, (2002) Distribution of chloroplast DNA variation in British oaks (*Quercus robur* and *Q. petraea*): The influence of postglacial colonisation and human management. *Forest Ecology and Management*, 156(1) 181-195.

Couvet, D. (2002) Deleterious effects of restricted gene flow in fragmented populations. *Conservation Biology*, 16(2) 369-376.

Dumayne, L. (1993) Invader or native? - vegetation clearance in northern Britain during Romano British time. *Vegetation History and Archaeobotany*, 2(1) 29-36.

EUFORGEN (2008a) *Distribution map of lime* (*Tilia cordata*). [map] www.euforgen.org. Available from http://www.euforgen.org/distribution_maps.html [Accessed 09-01-2012].

EUFORGEN (2008b) *Distribution map of lime* (*Tilia platyphyllos*). [map] www.euforgen.org. Available from http://www.euforgen.org/distribution_maps.html [Accessed 09-01-2012].

EUFORGEN (2011) EUFORGEN - *European forest genetic resources programme*. [online] Biodiversity International. Available from http://www.euforgen.org/ [Accessed 05-01-2012].

Fairbanks, R.G. (1989) A 17, 000-year glacio-eustatic sea level record: Influence of glacial melting rates on the younger Dryas event and deep-ocean circulation. *Nature*, 342(6250) 637-642.

Fineschi, S., Salvini, D., Taurchini, D., Carnevale, S. and Vendramin, G.G. (2003) Chloroplast DNA variation of *Tilia cordata* (*Tiliaceae*). *Canadian Journal of Forestry Research*, 33 2503-2508.

Fitch, S., Thomson, K. and Gaffney, V. (2005) Late Pleistocene and Holocene depositional systems and the palaeogeography of the Dogger bank, North Sea. *Quaternary Research*, 64(2) 185-196.

Friar, E.A., Boose, D.L., Ladoux, T., Roalson, E.H. and Robichaux, R.H. (2001) Population structure in the endangered Mauna Loa silversword, *Argyroxiphium kauense* (Asteraceae), and its bearing on reintroduction. *Molecular Ecology*, 10(7) 1657-1663.

Fromm, M. and Hattemer, H.H. (2003) Inheritance of allozymes and hybridization in two European *Tilia* species. *Heredity*, 91(3) 337-344.

Gibbons, E.J. (1975) *The flora of Lincolnshire*. 1st edition. Lincoln: Lincolnshire Naturalists' Union.

Godwin, H. (1940) Pollen analysis and forest history of England and Wales 1. *New Phytologist*, 39(4) 370-400.

Goldberg, E. and Kirby, K. (2002/3) *Ancient woodland: guidance material for local authorities*. [online] English Nature. Available from http://naturalengland.etraderstores.com/NaturalEnglandShop/UserFiles/Files/awg1.pdf [Accessed 14/12/11].

Grant, M.J., Waller, M.P. and Groves, J.A. (2011) The *Tilia* decline: Vegetation change in lowland Britain during the mid and late Holocene. *Quaternary Science Reviews*, 30(3-4) 394-408.

Gupta, S., Collier, J.S., Palmer-Felgate, A. and Potter, G. (2007) Catastrophic flooding origin of shelf valley systems in the English Channel. *Nature*, 448(7151) 342-345.

Haggett, G. (1997) The Limewoods of central Lincolnshire and their moths including Fletcher's Pug (*Eupithecia egenaria* H.-S. (Lep.: Geometridae). *The Entomologist's Record and Journal of Variation*, 109 287-292.

Hamrick, J.L., Mitton, J.B. and Linhart, Y.B. (1979) Levels of genetic variation in trees: Influence of life history characteristics. *Annual Review of Ecology & Systematics*, 10 173-200.

Hamrick, J.L., Godt, M.J.W. and Sherman-Broyles, S.L. (1992) Factors influencing levels of genetic diversity in woody plant species. *New Forests*, 6 95-124.

Hewitt, G.M. (1999) Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society*, 68 87-112.

Hosseinzadeh Colagar, A., Yusefi, M., Zarei, M. and Yousefzadeh, H. (2013) Assessment of genetic diversity of *Tilia rubra* DC. by RAPD analysis in the Hyrcanian forests, north of Iran. *Pol.J.Ecol*, 61(2) 341-348.

Kocer, Z.A., Gozen, A.G., Onde, S. and Kaya, Z. (2011) Indirect organogenesis from bud explants of *Juniperus communis* L.: Effects of genotype, gender, sampling time and growth regulator combinations. *Dendrobiology*, 66.

Lang, B., Bedford, A., Brooks, S.J., Jones, R.T., Richardson, N., Birks, H.J.B. and Marshall, J.D. (2010) Early-Holocene temperature variability inferred from chironomid assemblages at Hawes water, northwest England. *The Holocene*, 20(6) 943-954.

Liesebach, H. and Sinko, Z. (2008) A contribution to the systematics of the genus *Tilia* with respect to some hybrids by RAPD analysis. *Dendrobiology*, 59 13-22.

Lincolnshire Biodiversity Partnership (2011) *Lincolnshire biodiversity action plan 3rd edition.* [online]. Available from http://www.lincsbiodiversity.org.uk/docs/BAP/Lincolnshire%20BAP%203rd%20Edition/Lincs%20BAP%202011-2020%20FULL.pdf [Accessed 10-12-2011].

Lincolnshire Limewoods Project (2007) *Lincolnshire Limewoods*. [online]. Available from http://microsites.lincolnshire.gov.uk/limewoods/about/conservation/bardney-limewoods-nnr/ [Accessed 10-12-2011].

Logan, S.A., Phuekvilai, P. and Wolff, K. (2015) Ancient woodlands in the limelight: Delineation and genetic structure of ancient woodland species *Tilia cordata* and *Tilia platyphyllos* (*Tiliaceae*) in the UK. *Tree Genetics & Genomes*, 11(3) 1-12.

Loveless, M.D. and Hamrick, J.L. (1984) Ecological determinants of genetic structure in plant populations. *Annual Review of Ecology and Systematics,* 15 65-95.

Marcott, S.A., Shakun, J.D., Clark, P.U. and Mix, A.C. (2013) *A reconstruction of regional and global temperature for the past 11,300 years*. Science (New York, N.Y.), 339(6124) 1198-1201.

Muñoz-Concha, D. and Davey, M.R. (2011) Micropropagation of the endangered Chilean tree, *Gomortega keule*. *In Vitro Cellular & Developmental Biology,* 47(1) 170-175.

Nail, S. (2008) *Forest policies and social change in England*. Springer Science & Business Media.

Nair, L.G. and Seeni, S. (2003) In vitro multiplication of *Calophyllum apetalum* (Clusiaceae), an endemic medicinal tree of the Western Ghats. *Plant Cell, Tissue and Organ Culture*, 75(2) 169-174.

Natural England Bardney Limewoods, Lincolnshire. [online]. Available from https://designatedsites.naturalengland.org.uk/SiteDetail.aspx?SiteCode=S2000014&SiteName=bardney%20limewoods&countyCode=&responsiblePerson= [Accessed 15-07-15].

Natural England (2012) Sites of Special Scientific interest, SSSI information , Bardney Limewoods, Lincolnshire. [online] Natural England. Available from http://www.sssi.naturalengland.org.uk/Special/sssi/sssi_details.cfm?sssi_id=2000014 [Accessed 3/4/12].

Peterken, G.F. (1974) A method for assessing woodland flora for conservation using indicator species. *Biological Conservation*, 6(4) 239-245.

Peterken, G.F. (1996) *Natural woodland ecology and conservation in northern temperate regions*. 1st edition. Cambridge University Press.

Phuekvilai, P. (2014) *Relicts, refugia and reticulation: a study of population history, hybrids and phylogeny in the long-lived flowering tree genus Tilia*. PhD. Newcastle University.

Pigott, C.D. (1969) The status of *Tilia cordata* and *T. platyphyllos* on the Derbyshire limestone. *Journal of Ecology*, 57(2) 491-504.

Pigott, C.D. (1985) Selective damage to tree seedlings by bank voles (*Clethrionomys glareolus*). *Oecologia*, 67(3) 367-371.

Pigott, C.D. (1989) Factors controlling the distribution of *Tilia cordata* Mill at the northern limits of its geographical range. IV estimated ages of trees. *New Phytologist*, 112(1) 117-121.

Pigott, C.D. (1991) Biological flora of the British Isles. no 174 *Tilia cordata* Miller. *Journal of Ecology*, 79(4) 1147-1207.

Pigott, C.D. (1992) The clones of common lime (*Tilia*×*vulgaris* Hayne) planted in England during the seventeenth and eighteenth centuries. *New Phytologist*, 121(3) 487-493.

Pigott, D. (2012) *Lime-trees and basswoods: a biographical monograph of the genus Tilia*. Cambridge University Press.

Pigott, C.D. and Huntley, J.P. (1978) Factors controlling the distribution of *Tilia cordata* at the northern limits of its geographical range. I distribution in north-west England. *New Phytologist*, 81(2) 429-441.

Pigott, C.D. and Huntley, J.P. (1981) Factors controlling the distribution of Tilia cordata at the northern limits of its geographical range. III nature and causes of seed sterility. *New Phytologist*, 87(4) 817-839.

Provan, J. and Bennett, K.D. (2008) Review: Phylogeographic insights into cryptic glacial refugia. *Trends in Ecology & Evolution*, 23(10) 564-571.

Rackham, O. (1980) *Ancient Woodland: Its History, Vegetation and Uses in England.* Colvend: Castlepoint Press.

Rackham, O. (2001) *Trees and Woodland in the British Landscape*. 4th edition. Phoenix Press.

Rackham, O. (2008) Ancient woodlands: Modern threats. *New Phytologist,* 180 571-586.

Rackham, O. (2010) *Woodlands*. Collins.

Ratcliffe, D. (2012) *A nature conservation review: volume 1: the selection of biological sites of national importance to nature conservation in Britain*. Cambridge University Press.

Rhind, P. and Jones, B. (2003) The vegetation history of Snowdonia since the late glacial period. *Field Studies,* 10 539-552.

San José, M.C., Janeiro, L.V. and Corredoira, E. (2013) Micropropagation of threatened black alder. *Silva Fennica*, 47 1-12.

Selkoe, K.A. and Toonen, R.J. (2006) Microsatellites for ecologists: A practical guide to using and evaluating microsatellite markers. *Ecology Letters*, 9 615-629.

Smith, A.G. and Pilcher, J.R. (1973) Radiocarbon dates and vegetational history of the British Isles. *New Phytologist,* 72(4) 903-914.

Sturt, F., Garrow, D. and Bradley, S. (2013) New models of North West European Holocene palaeogeography and inundation. *Journal of Archaeological Science*, 40(11) 3963-3976.

Svejgaard Jensen, J. (2003) Technical Guidelines for genetic conservation and use for lime (Tilia spp). International Plant Genetic Resources Institute, Rome, Italy.: EUFORGEN.

Turner, J. (1962) The *Tilia* decline: An anthropogenic interpretation. *New Phytologist*, 61(3) 328-341.

Webster, C.A. and Jones, O.P. (1989) Micropropagation of the apple rootstock M.9 - effect of sustained subculture on apparent rejuvenation in vitro. *Journal of Horticultural Science,* 64(4) 421-428.

Weninger, B., Schulting, R., Bradtmoller, M., Clare, L., Collard, M., Edinborough, K., Hilpert, J., Joris, O., Niekus, M., Rohling, E.J. and Wagner, B. (2008) The catastrophic final flooding of Doggerland by the Storegga slide tsunami.. *Documenta Praehistorica*, 35(15) 1-24.

Wicksell, U. and Christensen, K.I. (1999) Hybridization among *Tilia cordata* and *T. platyphyllos* (*Tiliaceae*) in Denmark. *Nordic Journal of Botany*, 19(6) 673-684.

Yousefzadeh, H., Hosseinzadeh Colagar, A., Tabari, M., Sattarian, A. and Assadi, M. (2012) Utility of ITS region sequence and structure for molecular identification of *Tilia* species from Hyrcanian forests, Iran. Plant Systematics & Evolution, 298(5) 947-961.

CHAPTER TWO

Development of DNA Extraction Method and Initial Investigation into Genetic Diversity Using RAPD Markers.

2.0 Introduction

The investigation into the population structure of *Tilia cordata* (Mill.) within the Bardney Limewoods required the development of techniques that would improve or fully facilitate the identification of genetic variation between the closely located tree populations. Although many population studies use highly informative microsatellite markers to investigate genetic diversity, at the start of this investigation neither microsatellite markers, nor sequence data were available for *T. cordata* or any other *Tilia* sp.

For the initial pilot study, RAPD molecular markers were used to investigate the genetic population structure of two closely associated woods from within the Limewoods. To facilitate this, a DNA extraction protocol was developed that would provide nuclear DNA of suitable quality for use in subsequent PCR.

2.1 DNA Extraction

2.1.1 DNA Extraction Introduction

The extraction of high molecular weight DNA from plants can be problematic. The large differences in biochemical structure that exist between plants mean that DNA isolation protocols often have to be optimised for individual species. Common plant phytochemicals that can cause problems include polyphenols and polysaccharides. Any protocol developed has to yield sufficient DNA for subsequent procedures and, moreover, produce DNA of suitable quality for successful PCR analyses.

Polyphenols such as flavonoids, anthrocyanins, lignands and tannins are released from the cells and become oxidised when the tissue is ground during the initial stages of extraction. The resulting polyphenol oxidation products bind to and co-precipitate with the DNA and can inhibit enzymatic reactions, including subsequent PCRs. Strategies to remove or reduce the effect of polyphenolic compounds (Weising *et al*., 2005) include using polyphenol adsorbents, such as polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP) (Kasajima *et al*., 2013), the inclusion of phenoloxidase inhibitors, such as diethyldithiocarbamic acid (DIECA), using antioxidants, such as β-mercaptoethanol or ascorbic acid, to inhibit polyphenol oxidation (Stewart and Via, 1993; Borse *et al*., 2011) and, in addition, the dilution of the polyphenols by increasing the quantity of extraction buffer used.

Polysaccharides can also affect the PCR by inhibiting enzymatic reactions. Methods to reduce polysaccharide contamination of the DNA include (Weising *et al*., 2005) increasing the cetyltrimethylammonium bromide (CTAB) concentration in the extraction

buffer to bind the polysacarides in solution (Lundqvist *et al*., 2002), precipitating the DNA at room temperature to reduce co-precipitation of impurities (Michiels *et al*., 2003), removal of polysaccharides by their precipitation with alcohol in low salt concentration conditions (in this case the DNA remains in solution) (Michaels *et al*., 1994) and removal of DNA by precipitation with alcohol in high salt concentration conditions (in this case the polysaccharides remains in solution) (Fang *et al*., 1992; Kundu *et al*., 2011).

For *T. cordata*, Piggot (1991) found that all green parts contain mucilage ducts producing polysaccharides. *T. cordata* was also found to produce significant amounts of phenolic compounds when damaged during tissue culture and brown exudates from cut leaf tissue were observed in the culture medium. For these reasons, four different DNA extraction protocols were investigated, with the aim of minimising the effects of these compounds on subsequent PCRs.

2.1.2 DNA Extraction Materials and Methods

For all four methods of DNA extraction, tissue was taken from new *T. cordata* leaves that had been collected from buds which had been allowed to open under laboratory conditions and then preserved by freezing at - 70 ºC. Each DNA extraction was taken from a single *T. cordata* leaf of about 20 mm in diameter, providing approximately 0.26g of tissue for each extraction. Six samples were used for each extraction method and all the new leaf samples were taken from a single *T. cordata* tree from Great Scrubbs Wood (Lincolnshire) (individual tree identification Ch 2). Prior to extraction, the still-frozen tissue was ground to a fine powder in liquid nitrogen.

Four extraction protocols, utilising various methods to reduce the effects of polyphenol and polysaccharide contamination of the DNA, were compared; three using CTAB-based extraction buffers and one with an extraction buffer containing sodium dodecyl sulphate (SDS).

- CTAB B: a protocol modified by Stange *et al*. (1998) from a method by Stewart and Via (1993) incorporating CTAB, PVP, ascorbic acid and β-mercaptoethanol.
- CTAB I: a protocol incorporating CTAB, PVP and β-mercaptoethanol (Weising *et al*., 2005), together with steps to remove polysaccharides by their precipitation with alcohol in a low salt concentration (Michaels *et al*., 1994).
- CTAB II: a protocol developed by Weising *et al*. (2005) based on the method by Lassner *et al*. (1989).
- SDS - potassium acetate protocol (Dellaporta *et al*., 1983; Weising *et al*., 2005) using a high concentration potassium acetate in the presence of SDS to co-precipitate proteins and polysaccharides.

The protocols followed were taken from Weising *et al*. (2005) for the CTAB I, CTAB II, and the SDS- potassium acetate extractions, and from Stange *et al*. (1998) for the CTAB B extraction. The extraction buffers used for each of the protocols investigated are shown in table 2.1. Initially, RNA removal was not carried out for any of the methods.

The starting material for all four methods was approximately 0.26 g of fresh young leaf tissue. After extraction, electrophoresis of the rehydrated DNA, together with 200ng, 100ng and 50ng lambda phage and 100bp DNA ladder (New England Biolabs), enabled the DNA to be visualised, under ultra-violet light, on a 1% agarose gel containing 5µg/ml ethidium bromide. The quantity and size of the DNA produced from each method was estimated visually by comparison of the gel image intensity with that produced by lambda DNA (New England Biolabs) bands of 50 ng, 100ng and 200 ng DNA and 100 bp DNA ladder (New England Biolabs).

Table 2.1 Comparison of the four DNA isolation buffers used for the extraction of DNA from *T. cordata* leaf tissue.

	CTAB B (Stange *et al*. (1998) from a method by Stewart and Via (1993))	**CTAB I** (Weising *et al*., 2005)	**CTAB II** (Weising *et al.* (2005) based on Lassner *et al*. (1989))	**SDS-potassium acetate** (Dellaporta *et al*., 1983; Weising *et al*., 2005)
Hexadecyltrimethylammonium bromide (CTAB)	2.0%	2.0%	0.8%	
NaCl	1.42 M	1.4 M	800 mM	500 mM
EDTA pH 8	20 mM	20 mM	22 mM	50 mM
Tris-Cl pH 8	100 mM	100 mM	220 mM	100 mM
Ascorbic acid	5 mM			
Polyvinylpyrrolidone (PVP)	2.0%	1%	1%	
β-mercaptoethanol	0.3%	0.2%	0.2%	10 mM
Diethyldithiocarbamic acid (DIECA),	4 mM			
Sorbitol			140 mM	
Sarkosyl			1.0%	
Sodium dodecyl sulphate (SDS)				20%

Notes PVP and ascorbic acid added to buffer just before use.
β-mercaptoethanol added to individual extractions prior to incubation.
Concentrations are given either as molarity or as a percentage (CTAB, PVP, and SDS weight per volume, β-mercaptoethanol volume per volume).
(All chemicals supplied by Sigma Aldrich).

As a result of the extraction method comparison, the CTAB B protocol was further developed to be used for the subsequent molecular marker study. Modifications were made to the original protocol to improve the yield and aid the manipulation of the viscous DNA pellet. RNase A was introduced to remove RNA from the extract.

2.1.2.1 Optimised Extraction Method Based on the CTAB B Protocol

Where possible, *T. cordata* leaves that had been collected from buds which had been allowed to open under laboratory conditions were used as the starting material for the DNA extraction. For each tree sampled, a single *T. cordata* leaf of about 20 mm in diameter, providing approximately 0.26g of tissue, was used.

The leaf tissue was ground to a fine powder in liquid nitrogen and rapidly transferred to a 15 ml capped centrifuge tube containing 4 ml of CTAB extraction buffer (table 2.2). The sample was immediately incubated at 65 ºC for 30 minutes and mixed by inversion every 5 minutes. An equal volume of chloroform was added and the sample was mixed for 10 minutes before centrifuging at 6000rpm for 10 minutes at room temperature. To remove RNA, 750 µl of the top aqueous layer was taken from close to the chloroform buffer interface, transferred to a 1.5ml micro-centrifuge tube, together with 2.5 µl of 20 mg/ml RNAse A (100 mg/ml; 7000 units/ml; Qiagen), and incubated at 37 ºC for 30 minutes. An equal volume of chloroform-isoamyl alcohol (24:1) was added and the sample was mixed for 10 minutes before centrifuging at 10,000 rpm for 10 minutes at room temperature. The top aqueous layer was transferred to a new micro centrifuge tube with a wide-bored plastic Pasteur pipette to minimise shearing of the DNA. An equal volume of room temperature 100% isopropyl alcohol was added, the tube was mixed gently by inversion and the sample was either incubated at room temperature for 30 minutes or overnight

at -20 ºC to facilitate the precipitation of DNA. The mucilaginous DNA pellet was physically hooked out of the solution using the end of a pipette tip, transferred to a new tube and washed in 1ml of 70% ethanol. After washing, the DNA was again removed with a pipette tip to a new tube. The extracted DNA pellet was vacuum-dried for 10 minutes and resuspended in TLE buffer (10mM Tris (pH 8), 0.1mM EDTA (pH 8)). The volume of TLE used to rehydrate the samples was dependent on the size of the DNA pellet and varied between 0.1 and 1.0 ml to give a final DNA concentration of approximately 60 ng / µl.

The concentration and purity of the DNA extracted was estimated using a NanoDrop 1000 spectrophotometer (Thermo Scientific). After rehydrating, the extracted DNA samples were stored at -20ºC.

Table 2.2 CTAB isolation buffer developed for the extraction of DNA from *T. cordata* leaf tissue.

Hexadecyltrimethylammonium bromide (CTAB)	2.0 %
NaCl	1.42 M
EDTA pH 8	20 mM
Tris-Cl pH 8	100 mM
Ascorbic acid	5 mM
Polyvinylpolypyrrolidone (PVPP)	2.0 %
β-mercaptoethanol	0.3 %

Notes PVPP and ascorbic acid were added to buffer just before use.

β-mercaptoethanol was added to individual extractions prior to incubation.

Concentrations are given either as molarity or as a percentage (CTAB and PVPP weight per volume, β-mercaptoethanol volume per volume).

(All chemicals supplied by Sigma Aldrich)

2.1.3 DNA Extraction Results

All four methods successfully extracted DNA from approximately 0.26 g of leaf tissue (figure 2.1). The CTAB I, CTAB II and the SDS-potassium acetate methods all yielded less than 50 ng of genomic DNA / mg fresh leaf tissue for all samples. However, more DNA was obtained using the modified CTAB B extraction buffer method, where a yield of between 50 and 80 ng / mg of fresh leaf tissue was obtained. With no steps included to remove RNA, all four methods produced significant amounts of RNA.

As the protocol using the CTAB B extraction buffer gave the greatest DNA yield, this method was further modified to include steps to remove RNA and increase the purity of the resulting DNA. The effect of the removal of RNA from the extracted DNA is shown in figure 2.2. Quantification of the DNA using the Nanodrop spectrophometer gave an average yield of 0.22 μg / mg (s.d. 0.1) of fresh tissue with average 260/280 ratio = 1.9 and 260/230 ratio = 2.04.

The extraction method was found suitable for extraction from fresh plant tissue as well as tissue that had been frozen at -70 ºC or dried using silica gel. In all cases each DNA sample was extracted from a single *T. cordata* leaf of about 20 mm diameter, providing approximately 0.26 g of fresh leaf tissue, or 0.10 g of dry or frozen tissue. Where possible, young leaf tissue was obtained from buds that had been allowed to open under clean laboratory conditions.

Figure 2.1 The Comparison of four DNA extraction methods for *T. cordata*. Quantification of the genomic DNA extracted from approximately 260mg of *T. cordata* fresh leaf (10 µl loaded in each lane) can be estimated here by comparison with bands from 50 ng, 100 ng and 200 ng of lambda phage. Large amounts of RNA show up as bands with a size of < 500bp when measured against the included 100bp genomic ladder. All the samples were obtained from a single *T. cordata* tree from Great Scrubbs Wood in the Bardney Limewoods (individual tree identification Ch 2).

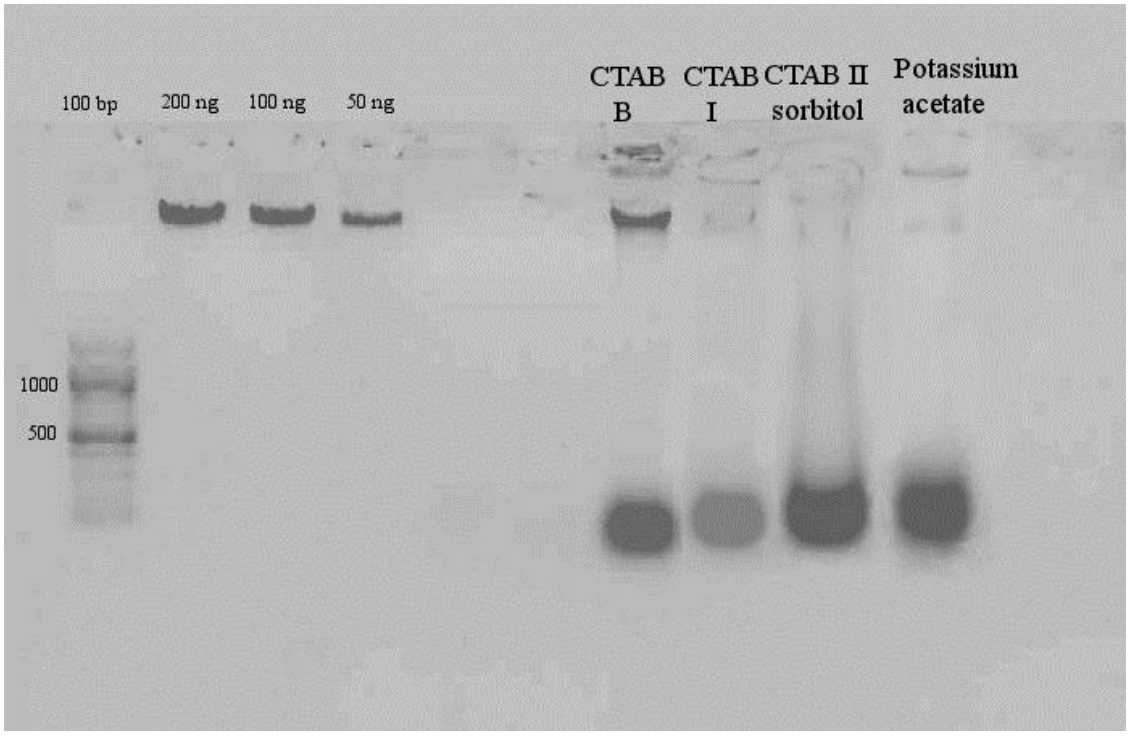
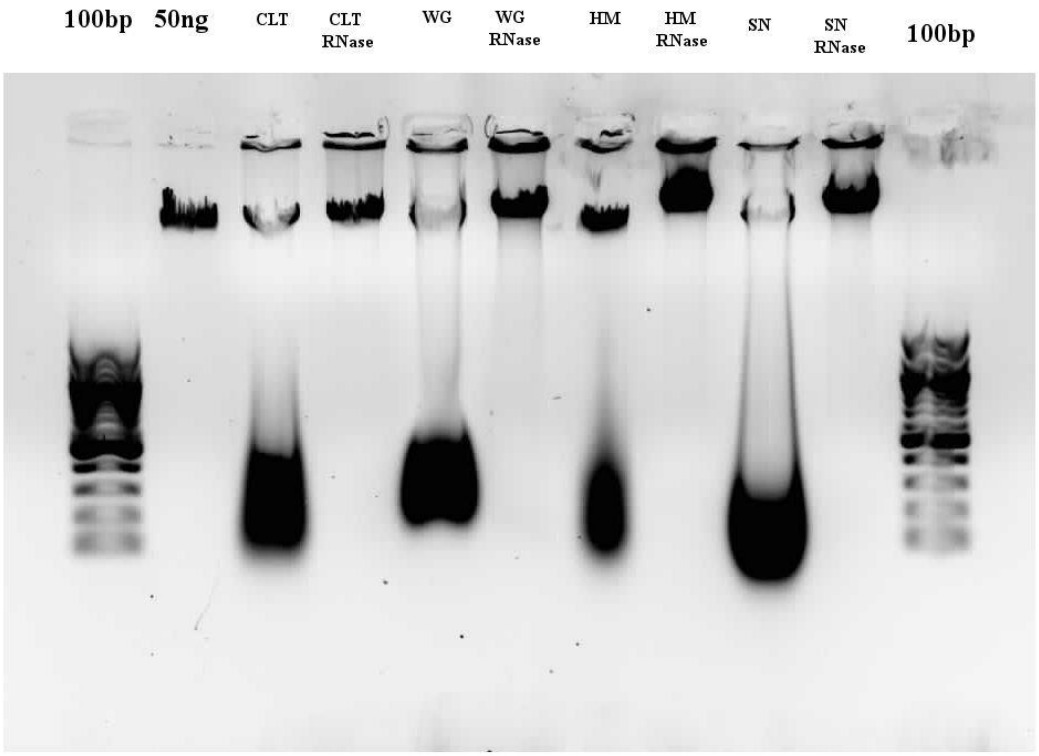


Figure 2.2 DNA extracted from *T. cordata*, using the modified CTAB B protocol, showing the effect of using RNase A to digest RNA. Genomic DNA was extracted from four separate trees taken from four of the Bardney Limewoods (Cocklode T (CLT), Wickenby G (WG), Hatton M (HM) and Southrey N (SN)). For individual trees, adjacent lanes compare DNA extracted with and without RNA removal. For the estimation of size and quantity of DNA extracted, lanes including 100 bp ladders and 50ng lambda phage DNA are also included.



2.1.4 DNA Extraction Discussion

Four DNA extraction protocols were initially investigated with the aim of optimising the method to produce DNA suitable for PCR analysis and also suited to analysing multiple samples. The protocols all included strategies to minimise the effects of polysacharrrides and polyphenols by co-precipitating contaminants that were likely to affect the result of PCRs (Maliyakal, 1992; Fang *et al.*, 1992).

Extraction of DNA from *T. cordata*, however, was initially found to be problematic as, during the initial cell disruption and lysis stage of DNA extraction, *T. cordata* leaves produced large amounts of viscous polysaccharides and this was particularly evident with older leaf tissue. This was consistent with the findings of Piggot findings (1991) that all green parts of *T. cordata* contain mucilage ducts producing polysaccharides. Polyphenols were also considered to be a potential problem during DNA extraction as *T. cordata* had been found to produce phenolic compounds when damaged during tissue culture, resulting in brown phenolic exudates from cut leaf tissue contaminating the culture medium (chapter 5).

Highly viscous polysaccharides were found to physically affect the extraction of DNA from *T. cordata* leaf tissue and consequently reduce the DNA yield from all the methods investigated. In the comparison described here, the CTAB B extraction method was found to give the highest yields of DNA (up to 80 ng / mg of fresh tissue when estimated visually) than for any of the other methods tested (all < 40 ng / mg of fresh tissue). This method, using the strategy of ethanol precipitation in a high salt CTAB extraction buffer, as suggested by Fang *et al.* (1992), to reduce polysaccharide contamination, was,

therefore, further developed to increase the yield and improve the purity of the DNA extracted.

The modified CTAB B buffer was formulated with high salt and CTAB concentrations, to reduce polysaccharide contamination, as well as 2 % polyvinyl polypyrrolidone (PVPP), which was found by Kasajima *et al*. (2013) to improve extraction efficiency. Ascorbic acid and β-mercaptoethanol were also included to reduce contamination from polyphenols. The initial volume of CTAB buffer to weight of tissue was also increased to increase the dilution of contaminants in solution. Other modifications were in relation to the large viscous DNA pellet that precipitated with the introduction of isopropyl alcohol to the extracted aqueous layer. This pellet was physically removed from the solution and washing it in 70 % ethanol was found to remove the viscous polysaccharides. RNA removal was also included in the extraction to improve PCR results.

The DNA yield using this method was improved to 0.22 µg / mg of fresh tissue (s.d. 0.1). The spectrophotometer 260/280 ratio of 1.9 and 260/230 ratio of 2.0 suggest that the DNA was pure with little polysaccharide or polyphenol contamination. To evaluate this extraction method, DNA was successfully amplified by the microsatellite primers developed for *T. cordata*.

2.2 RAPD Analysis

2.2.1 RAPD Analysis Introduction

A pilot study using Random Amplified Polymorphic DNA (RAPD) analysis (Williams *et al*., 1990) was carried out to investigate the genetic diversity of the populations of *T. cordata* found in the Bardney Limewoods. Four 10-mer primers, that had been reported as having been successfully used in the study of a variety of species, including plants (Arif *et al*., 2010), were used to investigate two small populations of *T. cordata*. Although RAPDs are dominant markers and are, therefore, not as informative as co-dominant markers such as microsatellites, they do not require DNA sequence information of the target species to be known. RAPD generates an array of DNA fragments, specific to the DNA being analysed, from the low-stringency PCR of short arbitrary primers that hybridise to compatible regions of the DNA of interest.

RAPD was chosen as it had proved useful in studies into species differentiation (Van de Ven and McNicol, 1995; Nkongolo *et al*., 2002) and has been employed in population studies, including the populations of a number of tree species. Aagaard *et al*. (1998) showed that, for six populations of Douglas fir from three subspecies, RAPDs and allozymes gave statistically similar results with, for RAPDs, 29% of the genetic variation occurring among Douglas fir subspecies and 8% occurring within the individual subspecies populations. Zarek (2009) used RAPDs to assess the genetic diversity of *Taxus baccata* L. within four natural populations in Poland. *T. baccata* populations from mountainous and lowland regions were differentiated and, as with many wind pollinated outcrossing species, most of the genetic variation was found to occur within the populations (variation within populations, 74%; variation between populations 26%).

A study using RAPDs carried out by Cottrell *et al*. (1997) identified low genetic diversity and widespread clonal duplication within the British population of black poplar, recognising the endangered status of this native species.

RAPD has also been used to study *Tilia spp*. Hosseinzadeh Colagar *et al.* (2013) studied six closely located (< 50km separation) populations of *Tilia rubra* D.C. in Iran and identified low inter-population differentiation between the groups, with 97% of the total genetic variation occurring within populations and 3% among populations. Liesebach and Sinkó (2008) investigated the characteristics of *Tilia* hybrids compared to various *Tilia spp*. using UPGMA cluster analysis of Dice similarity coefficients to elucidate relationships between the trees. It was, therefore, considered that RAPD was an appropriate marker to investigate differentiation of *T. cordata* at the population level.

2.2.2 RAPD Analysis Materials and Methods

Samples of unopened buds from *T. cordata* were collected from two woods, 1.4km apart, within the Bardney Limewoods. Eight samples were collected from Great Scrubbs Wood (Ch) and four from Goslings Corner Wood (G). Buds were allowed to open in clean laboratory conditions and young leaves were stored at -70 ºC until required. DNA was extracted from frozen tissue using the modified CTAB extraction method developed by Doyle and Doyle (1987, 1990). The DNA was diluted before use in the PCR to give a concentration of approximately 10 ng/µl. RAPD reactions were performed using the Ilustra Ready-To-Go™ RAPD Analysis Kit (GE Healthcare Life Sciences catalogue no. 27-9502-01) according to the manufacturer's instructions. Four 10-mer primers, supplied with the RAPD kit (table 2.3), were used in the analysis. These primers were reported as

having been successfully used in the study of a variety of species including plants (Arif *et al*., 2010).

Each 25 µl RAPD PCR was carried out in a 0.5 ml thin-walled tube and contained 25 pmol of a single RAPD primer, 50ng of template DNA (i.e. 5µl of 10 ng/µl extracted *T. cordata* DNA), ddH_2O and one analysis bead. Each RAPD Ready To Go™ analysis bead provided, when rehydrated, thermostable polymerase (AmpliTaq DNA polymerase and Stoffel fragment), 0.4 mM each dNTP, 0.1 mg/ml BSA and buffer (3 mM $MgCl_2$, 30 mM KCl and 10mM Tris, pH 8.3). Two *E. coli* controls were also included for each single primer to check the ability of the RAPD beads to amplify DNA. An Applied Biosystems® GeneAmp PCR System 9700 was used to facilitate PCR amplification and the thermal cycle used for the amplification was 95 ºC for 5 minutes, followed by 45 cycles of 95 ºC for 1 minute, 36 ºC for 1 minute and 72 ºC for 2 minutes. The DNA banding pattern resulting from the PCR was visualised under UV light on a 2% agarose gel stained with 0.5 µg/ml ethidium bromide with HindIII cut lambda as a molecular weight marker (New England Biolabs inc.).

The image was processed using GelCompar II software (Applied Maths NV, available from http://www.applied-maths.com.), and well-defined bands were scored as present (1) or absent (0). Pairwise Dice similarity coefficients were calculated and cluster analysis of the data was carried out using the unweighted pair group method with arithmetic averages (UPGMA). The results were bootstrapped over 100 replicates using the dendrogram construction utility DendroUPGMA ((Garcia-Vallve *et al*., 1999) available from http://genomes.urv.cat/UPGMA/).

2.2.3 RAPD Analysis Results

The four RAPD primers used for the pilot study amplified DNA from 12 *T. cordata* trees from two woodland groups and produced reproducible band patterns. An example of the banding pattern from primer 2 is shown in figure 2.3. A total of 60 bands, ranging in size from 300 to 2500 bp, were identified with 32 (53.3%) of the bands appearing polymorphic. The number of bands detected varied between 11 bands for primer 2, with 27.3% being polymorphic, and 22 bands for primer 6, with 68.2% being polymorphic. The average number of bands per primer was 15.0 and the average number of polymorphic bands 8.0 (53.3%) (table 2.3). When the results from all four primers were combined all the trees produced unique banding patterns.

The Dice similarity coefficients were used to derive the corresponding distance matrix for the samples (table 2.4). The mean genetic distance between all the trees was low (0.16, s.d. 0.07) with the greatest distances found to occur between trees from different wood groups and the smallest distances occurring between trees within the same wood. The most dissimilar trees were found to be Ch.1 from Great Scrubbs Wood and G1 from Goslings Corner Wood with 27 differing bands and genetic distance 0.30. Trees within wood groups were found to be more similar, with G3 and G2 from Goslings Corner Wood being the most similar with a difference occurring at only two bands and genetic distance of 0.02. Within each wood the genetic distance between trees ranged between 0.02 and 0.07 for Goslings Corner Wood and 0.04 and 0.19 for Great Scrubbs Wood (table 2.4).

Figure 2.3 A typical RAPD gel-banding pattern after amplification with primer 2 for *T. Cordata* from two woodland groups, Goslings Corner Wood (G) and Great Scrubbs Wood (Ch). HindIII cut lambda DNA (λ) is used as a molecular weight standard.

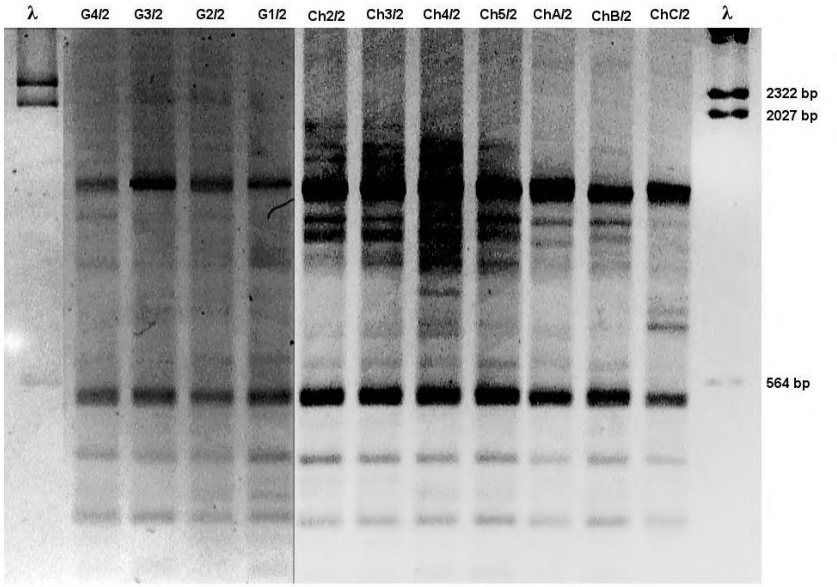


Table 2.3 RAPD primer sequences with number of bands, number of polymorphic bands and size range identified in the analysis of 12 *T. cordata* trees.

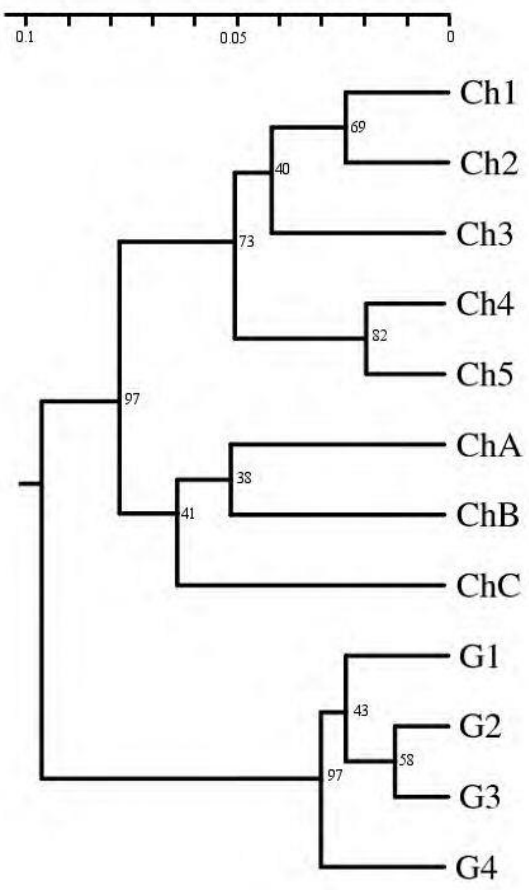
RAPD analysis primer	Primer sequence	No. of Amplified bands	No. of polymorphic bands	% of polymorphic bands	Fragment size range bp
1	GGTGCGGGAA	12	5	41.7	300-1000
2	GTTTCGCTCC	11	3	27.3	300-1100
3	GTAGACCCGT	15	9	60.0	300-1100
6	CCCGTCAGCA	22	15	68.2	300-2500
	Mean	15.0	8.0	53.3	

Table 2.4 Dice distance matrix based on the Dice similarity coefficient (Dice distance =1-Dice similarity coefficient). The values are derived from the combined RAPD scores of four primers used with DNA from 12 trees from two woods in the Bardney Limewoods. The two woods, Great Scrubbs Wood (Ch) and Goslings Corner Wood (G), are located approximately 1.4 km apart.

	Ch 1	Ch 2	Ch 3	Ch 4	Ch 5	Ch A	Ch B	Ch C	G1	G2	G3	G4
Ch 1		0.050	0.080	0.087	0.109	0.163	0.163	0.191	0.297	0.244	0.267	0.261
Ch 2			0.089	0.077	0.078	0.152	0.192	0.158	0.261	0.253	0.253	0.226
Ch 3				0.107	0.129	0.143	0.184	0.170	0.231	0.267	0.244	0.196
Ch 4					0.038	0.129	0.109	0.175	0.213	0.204	0.226	0.200
Ch 5						0.152	0.131	0.158	0.196	0.187	0.209	0.204
Ch A							0.104	0.152	0.169	0.205	0.182	0.156
Ch B								0.109	0.169	0.159	0.182	0.156
Ch C									0.200	0.190	0.190	0.163
G1										0.062	0.037	0.060
G2											0.025	0.073
G3												0.049
G4												

The UPGMA cluster analysis of the RAPD results, using distances derived from Dice similarity coefficients (figure 2.4), divide the trees into two main clusters that correspond to the two wood groups from which the trees were collected. Bootstrap values of the cluster analysis, over 100 replicates, indicates that these two main clusters are supported with bootstrap value of 97%. The Great Scrubbs Wood trees were further divided into two clusters (bootstrap, 97%) with ChA, B, and C separating from Ch1, 2, 3, 4 and 5. All other branches of the clusters were not so well supported, having bootstrap values of < 82%.

Figure 2.4 Cluster analysis, using Dice similarity coefficients, of the results from the RAPD analysis of *T. cordata*. The 12 trees were from two populations, eight trees from Great Scrubbs Wood (Ch) and four trees from Goslings Corner Wood (G). The numbers by the branches show the percentage of bootstrap analyses that support that branch (bootstrap values are derived from 100 replicates).



2.2.4 RAPD Analysis Discussion

In this pilot study of the genetic diversity of *T. cordata* using RAPD the four primers chosen successfully amplified DNA from the 12 *T. cordata* samples. By combining the results from the four primers each tree could be uniquely identified. UPGMA cluster analysis, based on Dice similarity coefficients, gave distances between all 12 trees of < 0.1 with distances of < 0.08 for trees from Great Scrubbs Wood and < 0.03 for trees from Goslings Corner Wood. Even though these results are derived from a very small sample and use different primers they are similar to the estimated distance values obtained by Liesebach and Sinkó (2008) from a small group of trees from the mixed forest of Pritzhagen, Germany (*T. cordata,* 0.18; *T. platyphyllos,* 0.22). Hosseinzadeh Colagar *et al.* (2013) also found low inter-population differentiation in the study of six closely located Iranian populations of *Tilia rubra*.

Cluster analysis, supported by bootstrap values of 97 %, placed the trees into two clusters that corresponded with the two population groups and also further divided the trees within Great Scrubbs Wood. Liesebach and Sinkó (2008) found UPGMA cluster analysis to be in good agreement with the taxonomic relationship between *Tilia spp.* and had used RAPD to investigate the putative character of *Tilia* hybrids. Subsequent study identified *T. cordata*, *T. platyphyllos* and their hybrids occurring within Great Scrubbs Wood (chapter 4) and the presence of clusters separating the Goslings Corner and Great Scrubbs trees may possibly be a result of the sampled trees being of hybrid origin and not be an indication of population membership.

Although RAPD was used for the pilot study it was subsequently considered that the development of a microsatellite library would be more informative and possibly provide

better intrapopulation data, as microsatellites are co-dominant, highly polymorphic molecular markers. Nybom (2004) reports that, for plants, estimates of within-population diversity derived from microsatellite data are almost three times higher than those derived from dominant markers whereas among-population diversity is generally similar. In the study of hybrid poplar cultivars by Rajora and Rahman (2003) the microsatellite markers used were found to be almost six times more informative than RAPD markers. In the comparison of molecular markers using barley accessions by Russell *et al*. (1997) and soybean by Powell *et al*. (1996), the highly polymorphic, co-dominant microsatellite markers were also found to be more informative than dominant RAPD. Russell *et al*. (1997) suggest microsatellites may be more suitable for population genetic studies, with RAPD being more suitable for assessing the genetic relationships between cultivars.

Even though RAPD was able to identify each individual tree, extending the study with RAPD primers was considered to be unlikely to provide suitably informative results for a genetic population study where most of the trees in the study are from closely grouped woods within Lincolnshire and were considered to be predominantly from one tree species. The co-dominant nature of microsatellites and their ability to identify rare and private alleles was considered to be better suited to deriving information from these populations.

2.3 References

Aagaard, J.E., Krutovskii, K.V. and Strauss, S.H. (1998) RAPDs and allozymes exhibit similar levels of diversity and differentiation among populations and races of Douglas fir. *Heredity,* 81(1) 69-78.

Arif, I.A., Bakir, M.A., Khan, H.A., Al Farhan, A.H., Al Homaidan, A.A., Bahkali, A.H., Al Sadoon, M. and Shobrak, M. (2010) Application of RAPD for molecular characterization of plant species of medicinal value from an arid environment. *Genetics and Molecular Research,* 9(4) 2191-9198.

Borse, T., Joshi, P. and Chaphalkar, S. (2011) Biochemical role of ascorbic acid during the extraction of nucleic acids in polyphenol rich medicinal plant tissues. *Journal of Plant Molecular Biology and Biotechnology,* 2(2) 1-7.

Cottrell, J.E., Forrest, G.I. and White, I.M.S. (1997) The use of RAPD analysis to study diversity in British black poplar (*Populus nigra* L. subsp. Betulifolia (Pursh) W. Wettst. (Salicaceae)) in Great Britain. *Watsonia,* 21(4) 305-312.

Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983) A plant DNA minipreparation: version II. *Plant Molecular Biology Reporter,* 1(4) 19-21.

Doyle, J.J. and Doyle, J.L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin,* 19(1) 11-15.

Doyle, J.J. and Doyle, J.L. (1990) Isolation of plant DNA from fresh tissue. *Focus,* 12(1) 13-15.

Fang, G., Hammar, S. and Grumet, R. (1992) A quick and inexpensive method for removing polysaccharides from plant genomic DNA. *BioTechniques,* 13(1) 52-55.

Garcia-Vallve, S., Palau, J. and Romeu, A. (1999) Horizontal gene transfer in glycosyl hydrolases inferred from codon usage in *Escherichia coli* and *Bacillus subtilis*. *Molecular Biology and Evolution,* 16(9) 1125-1134.

Hosseinzadeh Colagar, A., Yusefi, M., Zarei, M. and Yousefzadeh, H. (2013) Assessment of genetic diversity of *Tilia rubra* DC. by RAPD analysis in the Hyrcanian Forests, north of Iran. *Polish Journal of Ecology*, 61(2) 341-348.

Kasajima, I., Sasaki, K., Tanaka, Y., Terakawa, T. and Ohtsubo, N. (2013) Large-scale extraction of pure DNA from mature leaves of *Cyclamen persicum* Mill. and other recalcitrant plants with alkaline polyvinylpolypyrrolidone (PVPP). *Scientia Horticulturae*, 164 65-72.

Kundu, A., Sarkar, D., Bhattacharjee, A., Topdar, N., Sinha, M.K. and Mahapatra, B.S. (2011) A simple ethanol wash of the tissue homogenates recovers high-quality genomic DNA from *Corchorus* species characterized by highly acidic and proteinaceous mucilages. *Electronic Journal of Biotechnology*, 14(1) 10-11. Available from http://www.scielo.cl/scielo.php?script=sci_arttext&pid=S0717-34582011000100010 [Accessed 03/05/2015].

Lassner, M., Peterson, P. and Yoder, J. (1989) Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. *Plant Molecular Biology Reporter*, 7(2) 116-128.

Liesebach, H. and Sinkó, Z. (2008) A contribution to the systematics of the genus *Tilia* with respect to some hybrids by RAPD analysis. *Dendrobiology*, 59 13-22.

Lundqvist, H., Eliasson, A. and Olofsson, G. (2002) Binding of hexadecyltrimethylammonium bromide to starch polysaccharides. Part I: surface tension measurements. *Carbohydrate Polymers*, 49 43-55.

Maliyakal, E.J. (1992) An efficient method for isolation of RNA and DNA from plants containing polyphenolics. *Nucleic Acids Research*, 20(9) 2318

Michaels, S.D., John, M.J. and Amasino, R.M. (1994) Removal of polysaccharides from plant DNA by ethanol precipitation. *Bio Techniques*, 17 274-276.

Michiels, A., Van den Ende, W., Tucker, M., Van Riet, L. and Van Laere, A. (2003) Extraction of high-quality genomic DNA from latex-containing plants. *Analytical Biochemistry*, 315 85-89.

Nkongolo, K.K., Michael, P. and Gratton, W.S. (2002) Identification and characterization of RAPD markers inferring genetic relationships among pine species. *Genome*, 45(1) 51-58.

Nybom, H. (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Molecular Ecology,* 13(5) 1143-1155.

Pigott, C.D. (1991) Biological flora of the British Isles. no 174 *Tilia cordata* Miller. *Journal of Ecology*, 79(4) 1147-1207.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S. and Rafalski, A. (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding*, 2(3) 225-238.

Rajora, O.P. and Rahman, M.H. (2003) Microsatellite DNA and RAPD fingerprinting, identification and genetic relationships of hybrid poplar (*Populus* x *canadensis*) cultivars. *Theoretical & Applied Genetics,* 106(3) 470-477.

Russell, J.R., Fuller, J.D., Macaulay, M., Powell, W., Waugh, R., Hatz, B.G. and Jahoor, A. (1997) Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. *Theoretical and Applied Genetics,* 95(4) 714-722.

Stange, C., Arce-Johnson, P. and Prehn, D. (1998) Isolation of *Pinus radiata* genomic DNA suitable for RAPD analysis. *Plant Molecular Biology Reporter*, 16(4) 366-366.

Stewart, C.N.J. and Via, L.E. (1993) A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. *Bio Techniques,* 14 748-751.

Van de Ven, Wilhelmina T.G. and McNicol, R.J. (1995) The use of RAPD markers for the identification of sitka spruce (*Picea sitchensis)* clones. *Heredity,* 75 126-126.

Weising, K., Nybom, H., Wolff, K. and Kahl, G. (2005) *DNA fingerprinting in plants: Principles, methods and applications.* 2nd edition. USA: CRC Press.

Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, 18(22) 6531-6535.

Zarek, M. (2009) RAPD analysis of genetic structure in four natural populations of *Taxus baccata* from southern Poland. *Acta Biologica Cracoviensia Series Botanica*, 51(2) 67-75.

CHAPTER THREE

The Development of Microsatellite Markers for the Investigation of the Population Structure of *Tilia cordata* Miller (Malvaceae) within the Lincolnshire Limewoods

3.1 Introduction

Two native species of lime trees are found in the British Isles; the small-leaved lime, *Tilia cordata* Miller and the large-leaved lime *Tilia platyphyllos* Scopoli, with *T. cordata* being the most widely distributed (Pigott, 1991; 2012). *Tilia x europaea* L. (common lime), a hybrid of *T. cordata* and *T. platyphyllos*, is also found in Britain and occurs naturally where *T. cordata* and *T. platyphyllos* are found together, e.g. in the woods on the Derbyshire limestone (Pigott, 1969). Without flowers or fruits being present morphological identification of the species is problematic. In *T. cordata* the inflorescences are uniquely held erect whereas in all other *tilia* species the inflorescences are drooping (figure 1.11). Immature and shade leaves (figure 1.9) are atypical of the mature sun leaves found on flowering and fruiting shoots at the top of the canopy and in full sunlight and hybrids have features that are somewhat intermediate between the two species (Pigott, 1991).

After the last ice age, *T. cordata* colonised most of England from glacial refugia in Europe and pollen records show that by 6,500 BP *T. cordata* was one of the most numerous trees in the woodlands (Birks, 1989). The Lincolnshire Limewoods are highly biodiverse ancient woodlands that contain important populations of native *T. cordata* (Peterken, 1974). Although ancient in origin, the woods have been managed and exploited by their local communities over many centuries and this may have some influence on the genetic structure of the populations that are found today.

Variation in the genetic structure of *Tilia* has been investigated using various molecular markers. For studies into species differentiation and hybridisation, allozymes (Maurer and Tabel, 1995; Fromm and Hattemer, 2003), random amplified polymorphic DNA (RAPD)

(Liesebach and Sinko, 2008; Hosseinzadeh Colagar *et al*., 2013) and internal transcribed spacers (ITS) from nuclear ribosomal DNA (Yousefzadeh *et al*., 2012) have been used. Restriction fragment length polymorphisms (PCR-RFLP) was used by Fineschi *et al*. (2003) to study the European distribution of *T. cordata* chloroplast DNA haplotypes. More recently, microsatellites or simple sequence repeats (SSR) have been developed for *T. platyphyllos* and have been used in the detection of population history and genetic variation in *Tilia* (Phuekvilai and Wolff, 2013; Phuekvilai, 2014).

Microsatellites are tandem repeats of between one and six nucleotides generally found randomly distributed throughout the genome (Hancock, 1999). The microsatellite sequences undergo frequent mutations, predominantly a result of slippage and proofreading errors that occur during DNA replication, and this results in changes in the number of repeats (Eisen, 1999; Selkoe and Toonen, 2006). Polymerase chain reaction (PCR) (Mullis and Faloona, 1987) is used to detect and measure this variability in the microsatellite region using primers designed to match sections of the highly conserved regions that flank the tandem repeats. Microsatellites are highly polymorphic co-dominant neutral markers. Their high mutation rates tend to give rise to significant allelic diversity with many alleles per locus and, as co-dominant markers, they are also able to provide information about heterozygosity. Because of this diversity and high mutation rate, microsatellites are particularly suitable for the investigation of populations in the recent past i.e. 10 to 100 generations (Beaumont and Bruford, 1999; Selkoe and Toonen, 2006) and for investigations into ecological questions on a local level. However, they are less suitable for broad studies across taxonomic groups (Selkoe and Toonen, 2006).

As highly informative co-dominant markers, microsatellites have proved useful in many tree population studies where they have been used to identify closely related genotypes and to investigate genetic diversity in natural and managed populations e.g. *Quercus petraea* L. (Muir *et al*., 2004), *Populus nigra* L.(Vanden Broeck *et al*., 2004), *Prunus avium* L. (Vaughan *et al*., 2007), *Fraxinus excelsior* L. (Beatty *et al*., 2015; Sutherland *et al*., 2010), *Juniperus communis* L. (Provan *et al*., 2008) and *Castenea sativa* Mill. (Martin *et al*., 2010).

Microsatellite loci to facilitate the study of *T. cordata* within the Lincolnshire Limewoods were, after an initial pilot study with RAPD markers, considered to be the most suitable markers for this investigation. Unlike microsatellites, RAPD (Welsh and McClelland, 1990; Weising *et al*., 2005) does not require sequence information and therefore has lower development costs. Criticisms of the use of RAPD include problems with the reproducibility of results and the dominant nature of the marker (Lowe *et al*., 2004). Microsatellite markers also have disadvantages associated with them, including problems with amplification, which can result in some markers failing to amplify at all, resulting in null alleles, under estimation of genetic divergence due to homoplasy and difficulties that arise with the scoring of the results e.g. incorrect interpretation of stutter bands (Selkoe and Toonen, 2006; Chapuis and Estoup, 2007).

At the commencement of the study, microsatellites suitable for use with *T. cordata* were not available and the construction of an enriched microsatellite library was carried out to facilitate the development of this investigation. The microsatellites have been developed to study the genetic diversity and population structure of *T. cordata* and its potential hybridisation with *T. platyphyllos* within the Lincolnshire Limewoods. They will also be

used to compare the genetic diversity and structure in populations across England. Information about the population structure of the trees will inform and guide the current management policies that are used to conserve the biodiversity of the ancient woodlands and ensure their survival.

3.2 Materials and Methods

The *T. cordata* microsatellites were isolated using the method developed by Glenn and Schable (2005). DNA for the development of the microsatellite library was taken from a pooled sample extracted from five *T. cordata* trees from Great Scrubbs Wood in the Bardney Limewoods, Lincolnshire (table 3.1). DNA was extracted from fresh leaf tissue from the five trees using the modified CTAB B extraction buffer and the method as described in section 2.1.2.1 of this thesis. The five DNA samples were pooled and one phenol-chloroform-isopropyl alcohol (24:24:1 pH 8) (Fisher Scientific) extraction was used to increase the purity and concentration of the sample, following the protocol from Sambrook and Russell (2001a). The DNA was recovered by ethanol precipitation after the addition of 3 M sodium acetate to give a solution concentration of 0.3 M (Sambrook and Russell, 2001a). The final dried pellet was resuspended in TLE buffer to give a concentration of approximately 500ng/µl DNA.

Table 3.1 The location of the five Bardney Limewoods *T. cordata* trees in Great Scrubbs Wood that were used for the construction of the microsatellite library.

Bardney Limewoods, Lincolnshire
Great Scrubbs Wood

Tree i.d.	latitude	longitude
A	53.25371800	-0.27840937
L2	53.25001500	-0.28032674
1	53.25365600	-0.27845684
2	53.25371800	-0.27842435
10	53.25336000	-0.27854365

3.2.1 Microsatellite Library Development

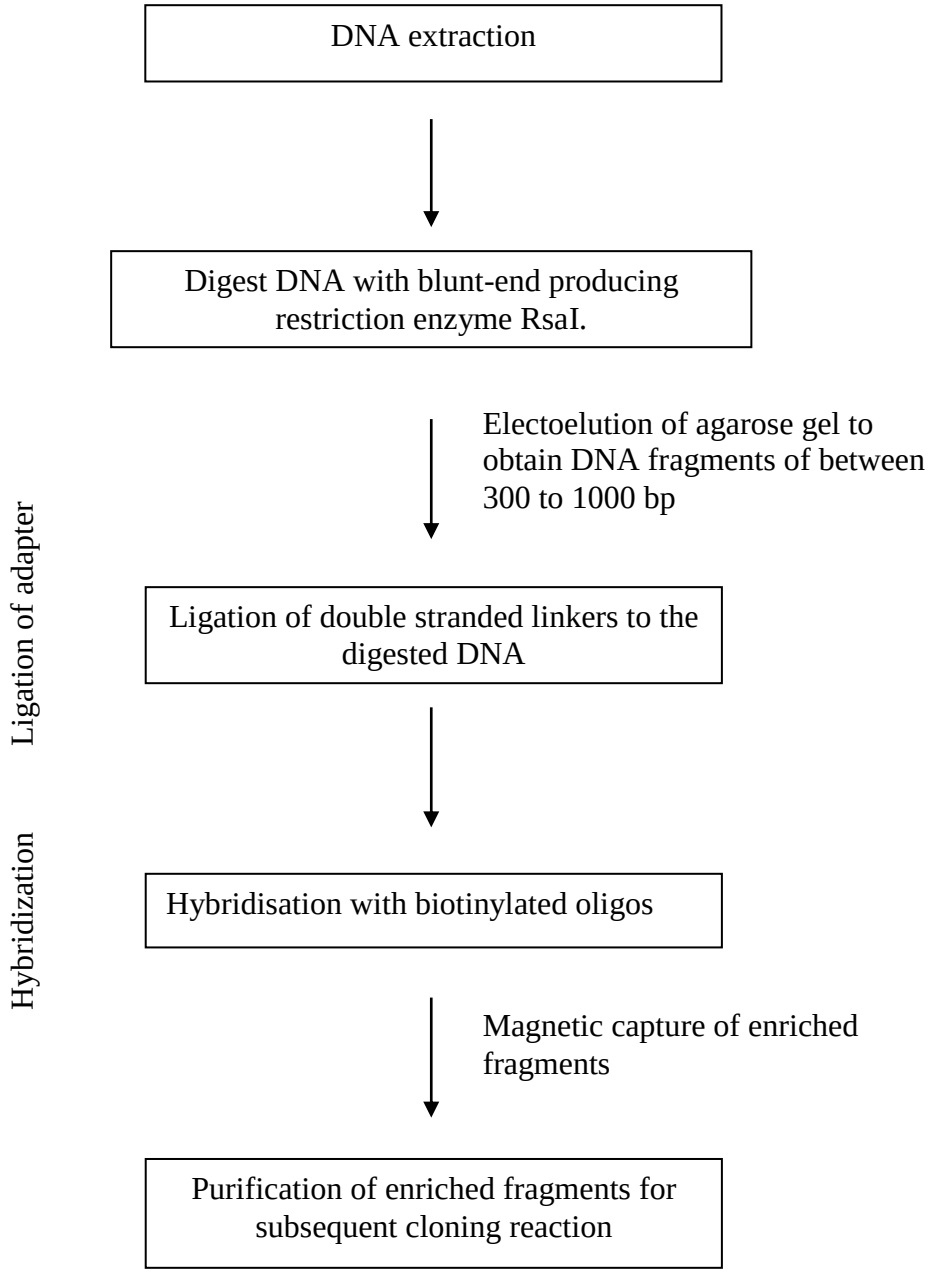
The microsatellite library for *T. cordata* was developed using the microsatellite enrichment protocol devised by Glenn and Schable (2005) (figure 3.1). To produce DNA fragments of a suitable size for the microsatellite library, *T. cordata* DNA (≈500ng/µl) was digested with the enzyme RsaI (New England BioLabs) for 5 hours at 35 ºC (10.0µl DNA (≈500ng/µl), 5.0µl Rsa1 10,000units/ ml (New England BioLabs), 4.0µl 10x ligase buffer (New England BioLabs) and 21.0µl H_2O). The Rsa I recognition site: -

5' G T ↓ A C 3'
3' C A ↑ T G 5'

cuts DNA to produce blunt ended fragments with a wide range of fragment sizes. As the range of fragment sizes obtained from this restriction enzyme digestion was greater than the required range of between 300 to 1000bp, the electroelution method in Sambrook and Russell (2001b) was used to select the required range of fragments sizes. The digested DNA was separated by electrophoresis in a 0.5 x TBE buffer on a 1% agarose gel stained with 0.5µg/ml ethidium bromide. Using 100 bp molecular ladder (NewEngland Biolabs), which was run concurrently with the digested DNA as a guide, a section of the DNA containing fragments in the range of 300 to 1000 bp was cut from the gel and placed inside a prepared viskine tubing bag containing a small amount of 0.25 x TBE buffer. The bag was placed parallel to the electrodes inside a gel tank containing 0.25 x TBE and an electric field of 7 volts/cm was applied between the electrodes for 60 minutes with the current reversed for the last 20s of the run. The TBE buffer containing the electroeluted DNA was removed from the bag. DNA was recovered from solution by ethanol precipitation. Sodium acetate (3M NaOAc pH 5.2) was added to the eluted DNA to give a concentration of 0.3M, 2 volumes of -20ºC ethanol were added, the solution centrifuged

and the supernant removed. The pellet of recovered DNA was washed in 70% ethanol, centrifuged, the supernant removed and the pellet dried before rehydrating in 45µl of TLE buffer. The size of the DNA fragments recovered from the gel was confirmed for the sample by electrophoresis on an agarose gel.

Figure 3.1 Summary of the microsatellite enrichment procedure.



The DNA fragments were ligated to linkers following Glenn and Schable's protocol (2005). Double-stranded (ds) SuperSNX linkers were prepared from the two primers

SuperSNX24 forward 5'GTTTAAGGCCTAGCTAGCAGAATC 3'
SuperSNX24+4P reverse 5'pGATTCTGCTAGCTAGGCCTTAAACAAAA

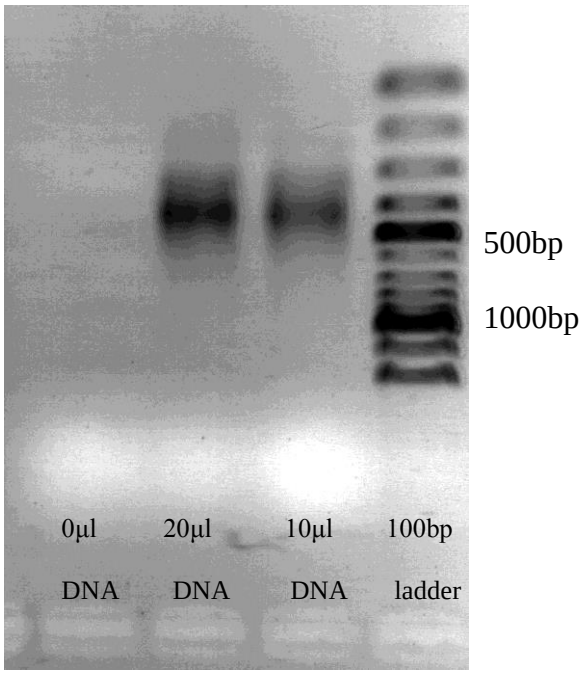
(Sigma Aldrich). The double stranded primers were ligated to the digested DNA in a reaction containing 20.0µl DNA, 7.0µl dsSuperSNX linkers, 1.0µl 10x ligase buffer, 2.0µl DNA ligase (NEB 400 units/ µl), 1.0µl XmnI (NEB), 0.25µl 100 x Bovine serum albumin (BSA), 2.5µl 10 x buffer 2 (NEB). The reaction was incubated at room temperature for 18 hours and the PCR product of the ligated fragments together with SuperSNX24 primer was observed on a 1% agarose gel to verify that the ligation was successful.

Enrichment of the fragments containing microsatellites was carried out by magnetic capture of the ligated DNA hybridized to a mixture of biotinylated oligos. The method developed by Glenn and Schable (2005) was followed to facilitate the capture of fragments containing microsatellite sequences complementary to those found in the introduced oligonucleotides. A mixture of biotinylate oligos $(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$ and $(ATC)_8$ (each oligo at 1.0µM (Sigma-Aldrich)) was hybridized to the ligated fragments. For the hybridization reaction, 10.0µl of linker ligated DNA, 25.0µl 2x Hyb solution (12x SSC, 0.2% SDS (stock solution of 20x SSC: 3.0 M NaCl, 0.3M sodium citrate, pH 7.0)), 10.0µl biotinylated microsatellite probe (mix of oligos at 1 µM each) and 5.0µl DI H_2O were incubated in a thermal cycler (Applied Biosystems® Veriti® 96-Well Thermal Cycler) to enable the oligonucleotides to hybridize with the DNA fragments. The thermal cycle (Applied Biosystems® Veriti® 96-Well Thermal Cycler) used was 95°C for 5 minutes, 70°C and steps down 0.2°C every 5 seconds for 99 cycles (i.e., 70°C for 5 sec, 68.8°C for 5 sec, 68.6°C for 5 sec, down to

50.2°C), 50°C for 10 minutes and then steps down 0.5°C every 5 seconds for 20 cycles (i.e., 50.0°C for 5 sec, 49.5°C for 5 sec, 49.0°C for 5 sec, down to 40°C), and finally 15°C.

Following the manufacturer's instructions, the biotinylated fragments were then allowed to bind to Dynabeads (Dynabeads®M-280 Streptavidin, Invitrogen) and concentrated using magnetic capture (Dynal MPC™-S, Invitrogen). A PCR of the purified enriched fragments was performed to verify the success of the enrichment process (figure 3.2).

Figure 3.2 The 1 % agarose gel image of the PCR product of the purified enriched fragments. Loading both 20 µl and 10 µl of the PCR product indicates that the fragments from the hybridisation and enrichment are mainly in the 500 to 300bp size range.



Using the protocol devised by Glenn and Schable (2005), the enriched DNA fragments were cloned to facilitate the identification of those containing microsatellites which were suitable for sequencing and analysis (figure 3.3). Using an Invitrogen TOPO-TA® cloning kit (Invitrogen, K4500-01), microsatellite-enriched DNA fragments were inserted into the linearised pCR™2.1-TOPO® Vector (figure 3.4) and transformed into chemically competent *E. coli* cells (One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen)) via a heat shock process. The transformed bacteria were grown on Luria-Bertani (LB) agar plates (1.0% tryptone (Oxoid), 0.5% yeast (Oxoid), 1.0% NaCl, 1.5% bacteriological agar (Oxoid)) containing 50µg/ml ampicillin and spread with Xgal (20mg/ml on each plate (Invitrogen)) and incubated at 37 ºC for 12 hours. After incubation, blue/white screening of the bacterial colonies enabled the selection of white colonies containing the recombinant DNA insert. The selected colonies were cultured individually at 37 ºC for 18 hours in 96 deep well plates with 25µl LB+amp broth (1.0% tryptone, 0.5% yeast, 1.0% NaCl, 50µg/ml ampicillin (Fisher)). The bacterial colonies grown in the LB broth were used as DNA template for PCR and 1.5µl of each white colony was amplified in a 25µl reaction volume (2.5µl BSA, 250µg/ ml; 2.5µl 10x PCR buffer; 1.0µl 10mM M13 forward primer; 1.0µl 10mM M13 reverse primer; 1.0µl 50mM MgCl; 1.5µl 2.5mM dNTPs (2.5mM of dGTP, dATP, dCTP, dTTP); 13.9µl DIH_2O (Sigma); 1.1µl Taq DNA polymerase (5 units/ µl)). The thermal cycle conditions (Applied Biosystems Veriti® 96 well thermal cycler) were 95 ºC for 3 minutes, 35 cyles of 95 ºC for 20s, 50 ºC for 20s, 72 ºC for 90s, a final extension of 72 ºC for 90s and hold at 15 ºC.

Figure 3.3 Summary of the cloning of restriction fragments enriched for microsatellites.

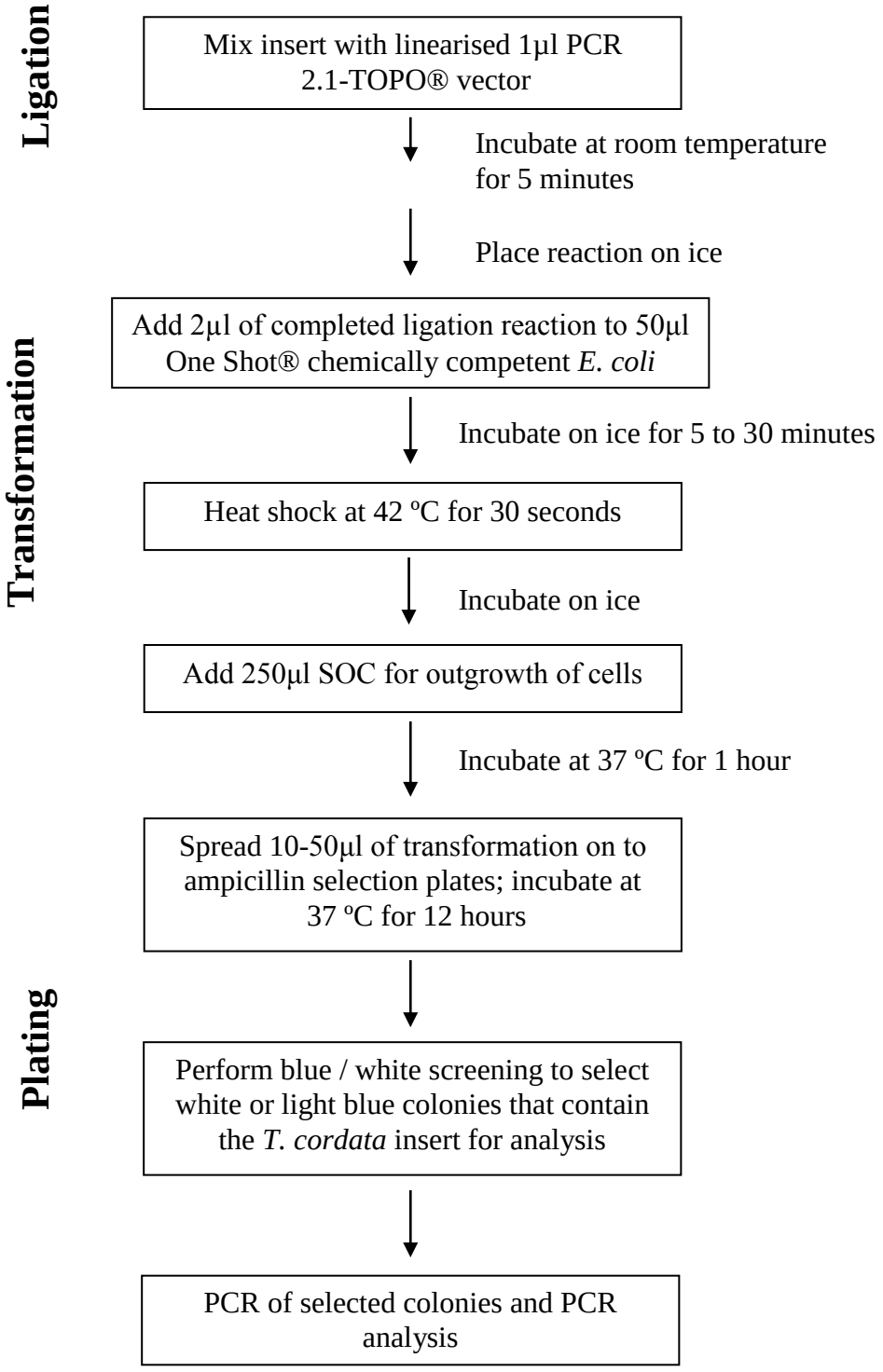
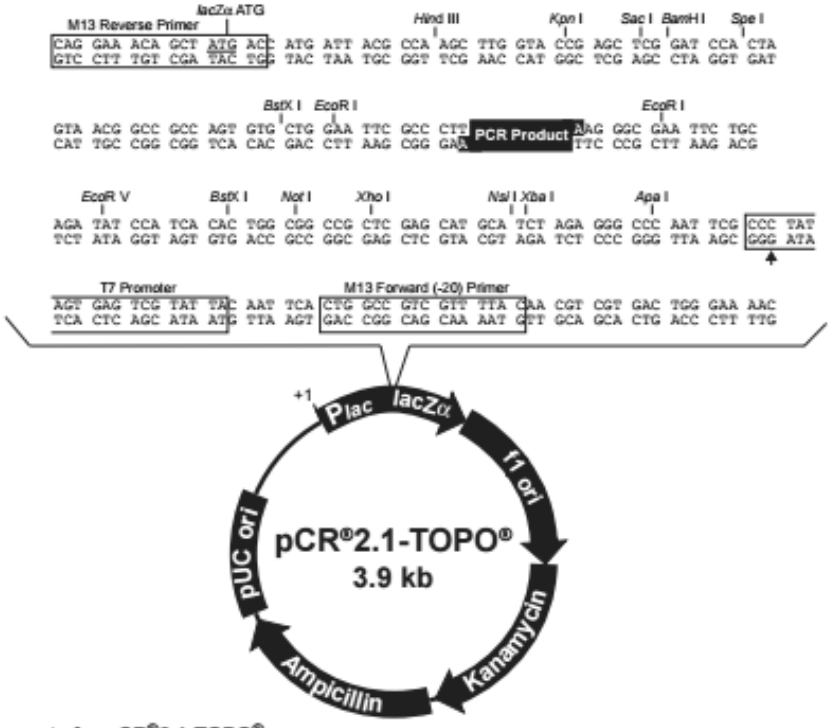


Figure 3.4 Cloning vector map for the pCR™ 2.1-TOPO® vector identifying the sequence surrounding the TOPO cloning site. The vector map indicates the position of the restriction and cleavage sites. The linearised vector is used in the cloning reaction. (www.lifetechnologies.com).



Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Invitrogen™
life technologies

https://tools.thermofisher.com/content/sfs/vectors/pcr2_1topo_map.pdf

The products of the PCR (3.0µl) were visualised on a 1% agarose gel containing 5µg/ml ethidium bromide and those in the size range of 500 to 1000bp were selected for sequencing. The chosen DNA fragments were cleaned using ExoSAP (1.0µl Exonuclease (ExoI) 10 units/ µl; 1.0µl shrimp alkaline phosphatase (SAP) 1unit/ µl) to remove unused primers and dNTPs. ExoSAP (0.5µl) was added to 5.0µl of each selected PCR product and incubated for 1 hour at 37 ºC and 15 minutes at 80 ºC. BigDye® Terminator v1.1 Cycle Sequencing Kit (Life Technologies, Applied Biosystems cat. no. 4337449) was used to generate samples for sequencing. For each reaction 2.0µl DNA from ExoSAP was used with 1.0µl M13 forward primer (1.6pM/ µl); 2.0µl sequencing kit mix (1.0µl BigDye sequencing kit, 0.5µl 5x sequencing buffer, 0.5µl DIH_2O) with the thermal cycling conditions 96 ºC for 1 minute; 25 cycles of 96 ºC for 10s, 50 ºC for 5s, 60 ºC for 240s and hold at 4 ºC. The PCR product was purified using an isopropanol clean up protocol. To each sample 90.0µl dilute isopropanol (13.5ml 100% isopropanol; 7.875ml DIH_2O) was added to give a final isopropanol concentration of 60 +/- 5%. The samples were vortexed, incubated at room temperature for 10 minutes and centrifuged at 13,000rpm for 30 minutes. The supernant was removed by aspiration. The DNA pellet was washed in 150µl of 70% isopropanol, mixed by inversion and centrifuged for 12 minutes at 13,000rpm. The liquid was removed by aspiration and the samples dried in a vacuum centrifuge for 10 minutes. The samples were stored dry at -20 ºC prior to sequencing.

The fragments obtained from the enriched library were sequenced in the forward direction by the Cardiff University DNA Sequencing Core on a 3130 XL Applied Biosystems Genetic Analyzer and analysed to detect di- and trinucleotide microsatellite sequences using Applied Biosystems Sequence Scanner Software v 1.0 (Applied Biosystems), Geneious v 6.1.2 (http://www.geneious.com, Kearse *et al.*, 2012) and ProSeq v 2.91

(Filatov, 2002). Primer 3 v 0.4.0 (Rozen and Skaletsky, 2000) was used to design primer pairs to flank the microsatellite region in the sequence. To optimise the yield of PCR product and reduce the likelihood of the non-specific binding of the primer sequence to the DNA, the default settings of Primer 3 were used together with an optimum GC content of 40%. As GC bonds are stronger than AT bonds, primers containing high GC content are more likely to mispair with other GC regions and produce non-specific DNA fragments during PCR. Net Primer (Premier Biosoft) was used to screen the selected primers for the potential to form secondary PCR structures, such as hairpins and cross- and self-dimers that could compromise the success of the primers to bind to specific DNA sequences. Optimal primer lengths of between 18 bp and 22 bp, melting point temperatures from 52 ºC to 58 ºC and GC contents of 40 % to 60 % were used to facilitate the design of primers that would be of sufficient length to uniquely identify the DNA sequences flanking the microsatellite, as well as being unlikely to produce fragments as a result of non-specific binding to the *T. cordata* DNA. A total of 19 primer pairs were constructed and tested for amplification using Qiagen Multiplex Kit (Qiagen cat. no. 206143) following the multiplex kit protocol for the amplification of microsatellite loci.

Primers that successfully amplified *T. cordata* DNA were labelled with either 6FAM, HEX (Sigma Aldrich) or NED (Applied Biosystems) to enable them to be used in multiplex PCR reactions. The volumes used for a total 6.0µl reaction volume with multiplexed primers were 3.0µl 2 x Qiagen PCR Multiplex Master Mix, 0.6µl 10x primer mix (to give a final concentration of each primer of 0.2µM), 1.2µl ddH_2O, 1.2µl DNA template (DNA≈ 1ng/µl). The thermal cycling conditions, using an Applied Biosystems® Veriti® 96-Well Thermal Cycler, were an initial activation step of 95 ºC for 15mins

followed by 35 cycles of 94 ºC for 30s, the appropriate primer anneal temperature (table 3.2) for 90s and 72 ºC for 60s, followed by a final extension of 60 ºC for 30mins.

After PCR the samples were prepared for analysis by the addition of HiDi™ formamide (Applied Biosystems) and the internal size marker 350 ROX™ (Applied Biosystems) (1.0µl PCR product; 10.0µl HiDi/ROX mix (960µl HiDi formamide, 48µl 350 ROX)). Genotyping was carried out by DNA Sequencing and Services at the University of Dundee on an ABI3730 capillary DNA sequencer (Applied Biosystems) and the data analysed using the computer software Geneious v 6.1.2 (http://www.geneious.com, Kearse *et al*., 2012). An internal size standard (GeneScan ROX 350 (Life Technologies)) was included in each sample and a separate allelic ladder derived from known fragments from selected trees was included on each PCR plate to enable consistent fragment size scoring across all the fragment plates.

3.2.2 Microsatellite Characterisation

The analysed microsatellite data was checked for the occurrence of null alleles using the programme Micro-Checker, which uses heterozygote deficiency to estimate the frequency of null alleles (Van Oosterhout *et al*., 2004) (available for download at http://www.microchecker.hull.ac.uk/).

The microsatellite loci developed were used to study the polymorphism of two population groups of *T. cordata*. These two populations both comprised of several small woods in close proximity; Chambers Farm Wood, Lincolnshire (number of trees N = 70, Latitude 53.25339 Longditude -0.27097732) and the Forest of Bere, Hampshire (N = 63, Latitude

50.911117, Longditude -1.1435701) (table 3.3). Statistical analysis was carried out using GENEPOP (Raymond and Rousset, 1995) and GenAlEx (Peakall *et al*., 2006; Peakall and Smouse, 2012).

The loci developed for use with *T. cordata* were also tested for their ability to amplify DNA from other closely related *Tilia* species, in particular *T. platyphyllos*. The genetic diversity of two small *T. platyphyllos* populations was investigated using a natural population from Wenlock Edge, Shropshire (N = 7, Latitude 52.578223, Longitude -2.6134076) and a planted avenue of trees in Riseholme Park, Lincolnshire (N = 8, Latitude 53.265525, Longitude -0.52547411) (table 3.4). The diversity of the *T. platyphyllos* populations was compared with that of the *T. cordata* populations. The microsatellite loci were also tested with *Tilia x euchlora* (N = 3) and *Tilia tormentosa* Moench (N = 1) collected from Riseholme Park.

To estimate the differentiation between *T. cordata* and *T. platyphyllos* the allele frequency for all ten microsatellite loci was compared for the two species using the complete data set of all sampled *T. cordata* and *T. platyphyllos* trees.

3.3 Results

From the 284 clones obtained from the enriched library, sequencing identified microsatellites in 48 of the fragments (16.9%) with five clones occurring more than once, giving a redundant clone occurrence of 25%. After screening for secondary PCR structures, 19 primer pairs were constructed and tested for the ability to amplify *T. cordata*. Five of the 19 tested primer pairs had fragment peak patterns that could not be resolved and were not analysed further. One primer pair (tc1-10) was not used in the study as it was monomorphic in both *T. cordata* and *T. platyphyllos* populations as well as for *T. x euchlora* and *T. tormentosa* trees, producing just one allele at 152bp.

The analysis of the data from *T. cordata* trees indicated that null alleles were likely, as a result of heterozygote deficiency, in six out of the remaining 13 polymorphic loci. Loci tc1-50, tc1-52 and tc1-70 were removed from further analysis at this point as they were identified as containing null alleles, with a mean null allele frequency of 0.32 and between 25 % and 62 % of missing data. Null alleles were also identified in loci tc1-45 (null allele frequency 0.06), tc2-86 (0.10) and tc2-07 (0.09). For the small *T. platyphyllos* population, after the removal of tc1-50, tc1-52 and tc1-70, possible null alleles were identified at tc1-30 (null allele frequency 0.05).

After optimisation, ten microsatellite loci were suitable for the *T. cordata* study. These ten loci and the monomorphic primer tc1-10 are characterised by primer sequence, observed allele size range, repeat motif and anneal temperature (table 3.2).

Table 3.2 Characterisation of 11 microsatellite loci cloned for *T. cordata*. The forward (F) and reverse (R) primers sequences are shown together with their observed allele size range, repeat motive and anneal temperature (Ta).

Locus	Observed allele size range bp	Repeat motif		Primer sequence 5' to 3'	Ta (ºC)
tc1-10	152	$(CAT)_5$	F	TCTGTGTTGTGGTTTTCTTTGC	58.1
			R	TCCCACTAATTGGTTTGTCG	
tc1-19	228 – 243	$(CAT)_2(CGT)(CAT)_6$	F	GCCGTCATTTTGATCCTCAT	57.8
			R	AGGTGGCGGTAATGTTTGT	
tc1-30	230 – 250	$(TC)_7$	F	CAAGTCAGCCTGTTGGGAAT	59.2
			R	CGAGGGATTGGTTCAATCAG	
tc1-42	120 – 152	$(AG)_{13}$	F	TGGTATGCCACACGGAGATA	58.5
			R	TGCACCTTCCAGATCCAA	
tc1-45	165 – 249	$(TGA)_6(GGA)_3$	F	CACCTCAAGTGATTGTGCCTTA	58.9
			R	TTGCTCACCTTGTGCTATCG	
tc2-07	242 – 260	$(TG)_8$	F	CATCTTTTCTCCCCCATTCA	58.2
			R	CCAAAGGCATAACAGAACAAAA	
tc2-16	214 – 242	$(GT)_9$	F	CAAGATGCACATGAGGCACTA	58.7
			R	GAAATTGGGCAGGCTGTAAG	
tc2-69	226 – 292	$(GA)_{12}(N)_{33}(GA)_3$	F	AGCTGAACACAACCTGCAAA	58.9
			R	ATTGATGGCTCGCTCATCTC	
tc2-86	215 – 233	$(GAT)_6$	F	CCCACTGTCCCCCAATATGT	59.5
			R	TTCGCCTTGTTTCCTGATTT	
tc3-57	166 – 196	$(GT)_{10}(GA)_{11}$	F	CTGGCTAAGTTGCCCATAAA	57.8
			R	TGTCCTTGCTGCTTGCTCTA	
tc3-74	140 – 172	$(GA)_9$	F	ATGACGAATTCCATCCCAGA	57.2
			R	CGCAGACGTTATAGAAGCC	

3.3.1 Genetic Diversity and Cross Amplification between Species

The ten primers were tested with two populations of *T. cordata*, a Lincolnshire population from Chambers Farm Wood and a population from the Forest of Bere in the south of England (table 3.3). The ten microsatellite primers were found to be highly polymorphic in both *T. cordata* populations. For the ten polymorphic loci the mean number of alleles (mean Na) found per locus was 4.8 (s.e. 0.71) for Chambers Farm Wood, with a range of between 2 and 8 alleles, and 4.6 (s.e.0.54) for the Forest of Bere, with the same range. The variation in mean observed and expected heterozygosity was Ho = 0.48 (range 0.87 to 0.06), He = 0.52 (range 0.82 to 0.06) for Chambers Farm Wood and Ho = 0.47 (range 0.71 to 0.09), He = 0.53 (range 0.79 to 0.11) for Forest of Bere. The most diverse loci for both of the Chambers Farm Wood and Forest of Bere populations was tc3-57.

A t-test showed that there was no significant difference between the two populations for mean number of alleles, mean Na (P = 0.85), effective number of alleles, Ne (P = 0.77), Ho (P = 0.89) or He (P = 0.94). Significant deviations from Hardy-Weinberg equilibrium (HWE) occurred in both populations at three loci, tc2-69, tc2-86 and tc3-74, with tc2-69 being highly significant in both populations ($P < 0.001$). Two other loci showed deviations from HWE for just one of the populations (tc1-42 at Chambers Farm Wood and tc2-16 Forest of Bere, both $P<0.05$).

Table 3.3 General genetic diversity estimates for two *T. cordata* populations (Chambers Farm Wood, Lincolnshire and Forest of Bere, Hampshire) from the analysis of 11 microsatellites.

	Chambers Farm Wood N = 70					Forest of Bere N = 63				
Locus	Na	Ne	Ho	He	HWE[1]	Na	Ne	Ho	He	HWE[1]
tc1-10	1	1	monomorphic			1	1	monomorphic		
tc1-19	3	2.71	0.691	0.631	ns	4	3.05	0.636	0.672	ns
tc1-30	8	4.07	0.754	0.754	ns	6	2.69	0.581	0.628	ns
tc1-42	6	2.22	0.586	0.55	*	5	2.18	0.476	0.542	ns
tc1-45	2	1.37	0.203	0.268	ns	2	1.49	0.254	0.328	ns
tc2-07	2	1.42	0.269	0.294	ns	3	2.06	0.473	0.515	ns
tc2-16	3	1.06	0.057	0.056	ns	5	1.12	0.095	0.107	*
tc2-69	6	4.14	0.455	0.759	***	5	3.74	0.571	0.733	***
tc2-86	5	2.68	0.522	0.627	***	5	2.55	0.54	0.608	*
tc3-57	8	5.6	0.868	0.822	ns	8	4.8	0.707	0.791	ns
tc3-74	5	1.93	0.414	0.481	*	3	1.67	0.349	0.402	**

Table 3.4 General genetic diversity estimates for two *T. platyphyllos* populations (a planted avenue in Riseholme Park, Lincolnshire and a natural population from Wenlock Edge, Shropshire) from the analysis of 11 microsatellites.

	Riseholme *T. platyphyllos* N = 7					Wenlock Edge *T. platyphyllos* N = 5				
Locus	Na	Ne	Ho	He	HWE[1]	Na	Ne	Ho	He	HWE[1]
tc1-10	1	1	monomorphic			1	1	monomorphic		
tc1-19	3	2.39	0.429	0.582	**	3	2.63	0.8	0.62	ns
tc1-30	5	4.45	1	0.776	**	3	2.13	0.5	0.531	ns
tc1-42	2	1.85	0.429	0.459	ns	3	2.67	0.5	0.625	ns
tc1-45	3	2.88	0.5	0.653	*	3	2.91	0.25	0.656	ns
tc2-07	2	2	1	0.5	ns	3	2.57	1	0.611	ns
tc2-16	1	1	monomorphic			2	1.28	0.25	0.219	ns
tc2-69	5	4.26	0.857	0.765	ns	5	4	0.75	0.75	ns
tc2-86	3	2.65	1	0.622	ns	1	1	monomorphic		
tc3-57	3	2.65	1	0.622	ns	4	1.92	0.6	0.48	ns
tc3-74	3	2.65	0.571	0.622	ns	1	1	monomorphic		

Key for tables 3.3 and 3.4
N = no. of individuals, Na = no. of alleles, Ne = no of effective alleles,
Ho = observed heterozygosity, He = expected heterozygosity,
HWE Hardy-Weinberg equilibrium,
HWE[1] ns = not significant, * P <0.05, ** P <0.01, *** P <0.001

The ability of the primers to amplify other *Tilia* species was also tested. All the selected primers successfully amplified DNA taken from trees in two small groups of *T. platyphyllos*, a naturally occurring population of trees from Wenlock Edge, Shropshire (N = 7, Lat 52.578223, Long -2.6134076) and a planted avenue of trees in Riseholme Park, Lincolnshire (N = 8, Lat 53.265525, Long -0.52547411). *T. tormentosa* (N = 1) and *T. x euchlora* (N = 3), planted trees from the Riseholme Park collection, were also found to be amplified by all of the primers.

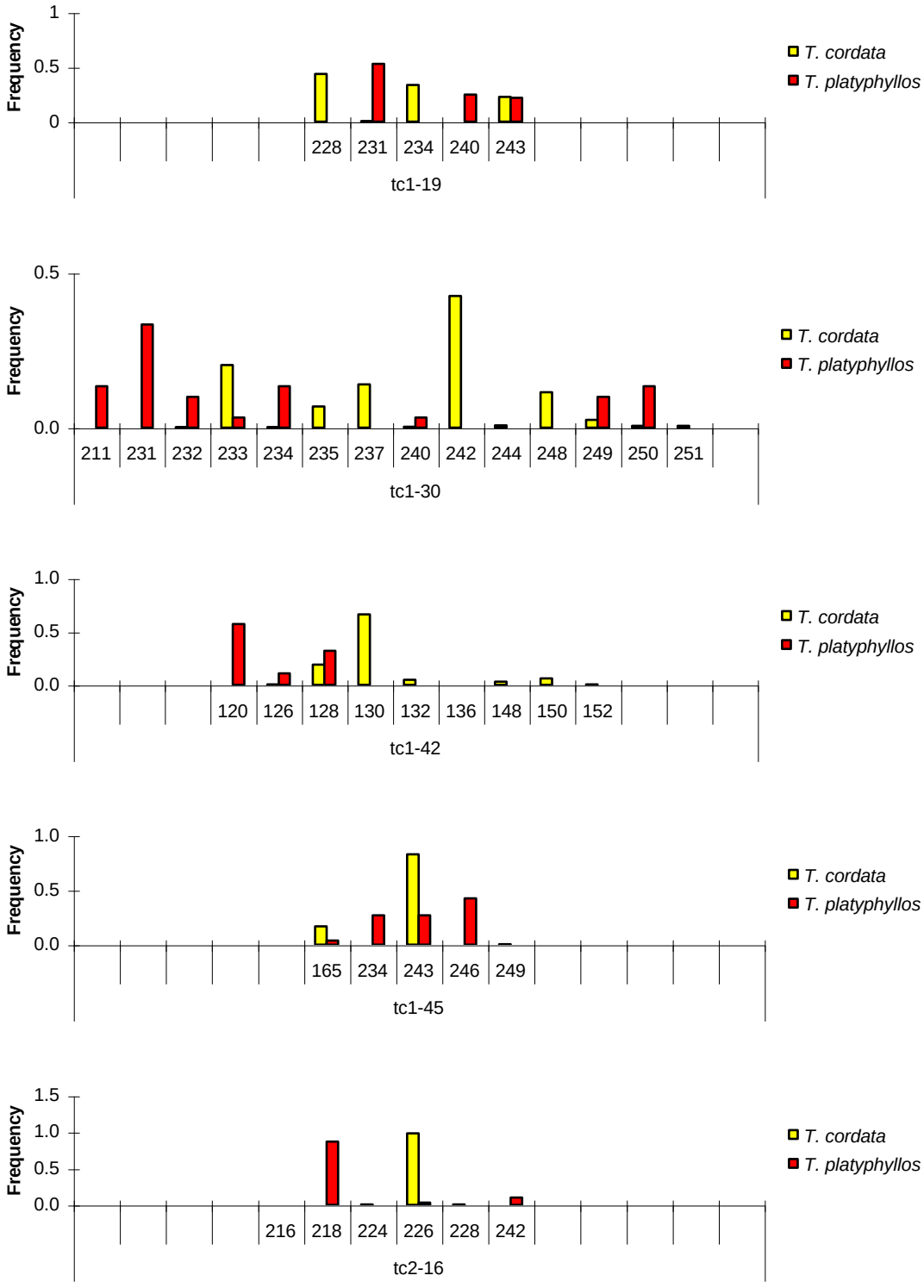
T. platyphyllos was monomorphic for tc1-10, as was *T. cordata*, but the two small populations were also monomorphic for tc2-16 (Riseholme) and tc2-86 and tc3-74 (Wenlock Edge) (table 3.4). For the polymorphic loci, the mean number of alleles (Na) found per locus was 3.0 (s.e. 0.39) for the Riseholme trees, with a range from one to five alleles, and 2.8 (s.e. 0.39) for the Wenlock Edge natural population, also with a range from one to five alleles. The variation in mean observed and expected heterozygosity was, for Riseholme Ho = 0.68 range 0.86 to 0.43, He = 0.56 range 0.76 to 0.46 and for the Wenlock Edge trees Ho = 0.46 range 0.8 to 0.25, He = 0.45 range 0.75 to 0.22. The most diverse loci were tc1-30 and tc2-69 for the Riseholme and Wenlock Edge populations respectively.

A t-test showed that there was no significant difference between the Riseholme and Wenlock Edge populations for mean Na (P = 0.75), Ne (P = 0.35), Ho (P = 0.19) or He (P = 0.36). Nor was there any significant difference between *T. cordata* and *T. platyphyllos* when the species themselves were considered as separate groups mean Na (P = 0.53), Ne (P = 0.24), Ho (P = 0.08) and He (P = 0.15).

Significant departure from Hardy-Weinberg equilibrium (HWE) for both the Chambers Farm Wood and the Forest of Bere populations was observed at tc2-69 ($P < 0.001$, $P<0.001$), tc2-86 ($P <0.001$, $P = 0.045$) and tc3-74 ($P = 0.015$, $P = 0.008$). Significant departure from HWE was also observed at tc1-42 ($P = 0.024$) but, in this case, only at Chambers Farm Wood. When the loci were used for the small *T. platyphyllos* populations, significant departures from HWE were only observed in the planted Riseholme Park trees, at tc1-19 ($P = 0.002$), tc1-30 ($P = 0.002$) and tc1-45 ($P = 0.028$).

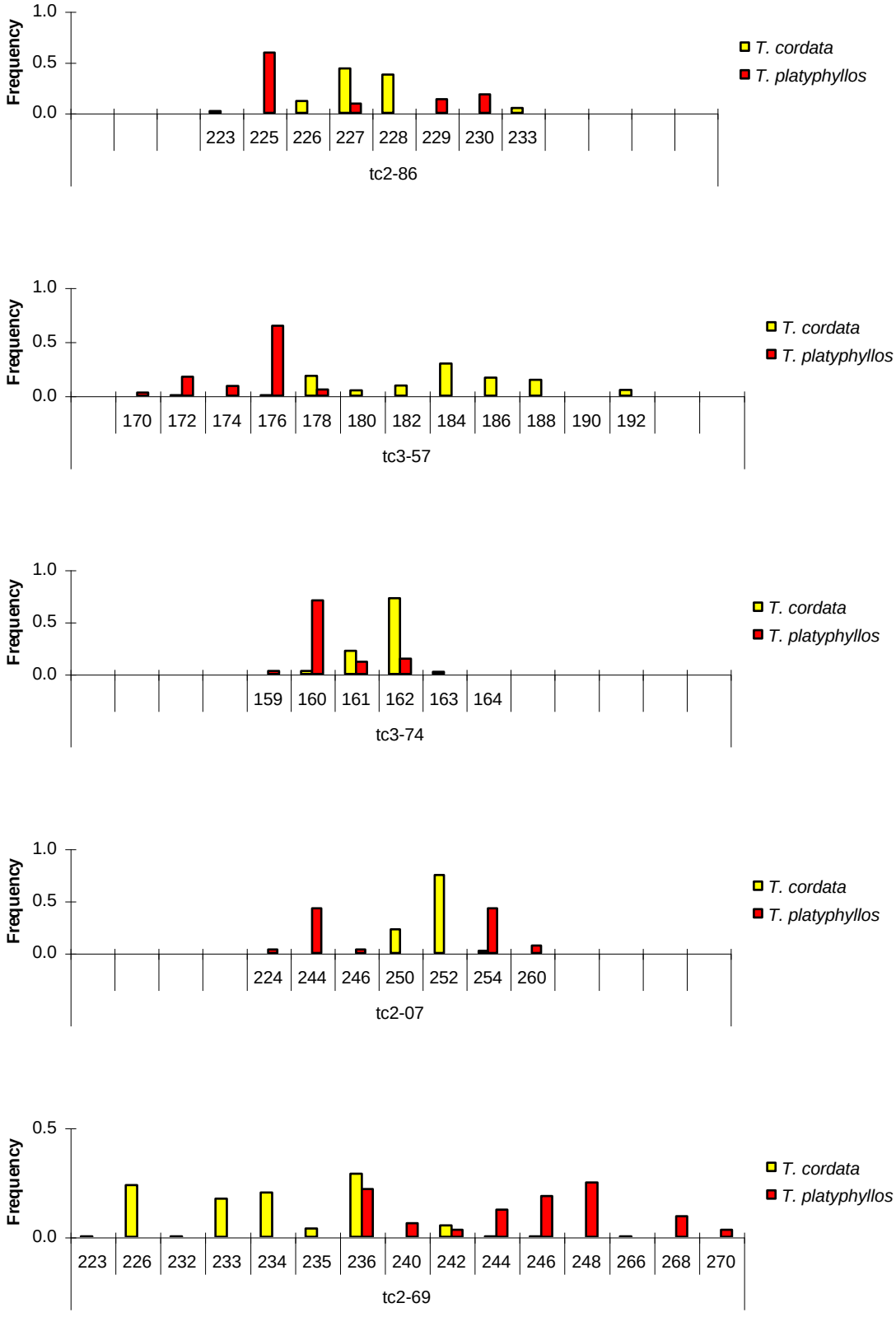
Using data from all the trees sampled for the study, a comparison was made of the allele frequency of *T. cordata* (N = 392) and *T. platyphyllos* (N = 17) (figure 3.5). From the ten microsatellite loci, a total of 90 alleles were identified. 34 alleles were exclusive to *T. platyphyllos* and 25 to *T. cordata*, with 23 alleles shared between *T. cordata* and *T. platyphyllos*. The allele ranges for *T. cordata* and *T. platyphyllos* were shared for all 10 loci but for tc3-57 the range overlapped at only one allele, 178bp (*T. platyphyllos* 5 alleles 170 to 178bp; *T. cordata* 7 alleles 178 to 192bp). *T. cordata* is monomorphic at the locus tc2-16 (226bp) and *T. platyphyllos* has three alleles but only the one at 218 bp occurs at high frequency.

Figure 3.5 Allele frequency for each of ten microsatellite loci with the distribution of alleles indicated for populations of both *T. cordata* and *T. platyphyllos*.



(Continued)

Figure 3.5 (continued) Allele frequency for each of ten microsatellite loci with the distribution of alleles indicated for populations of both *T. cordata* and *T. platyphyllos*



3.4 Discussion

From an initial 286 clones, 19 *T. cordata* microsatellite markers were designed using an enrichment protocol using hybridisation capture. The olignuclotides used to enrich the microsatellites are chosen without knowledge of the actual abundance and structure of the microsatellites within the *T. cordata* genome. As a result selection bias can be introduced into the genomic library (Leese *et al*., 2008) and this can effect how representative the microsatellites are of the actual distribution within the genome (Duan *et al*., 2014). This can also be problematic if the loci are tested in other related species, as the distribution of polymorphism not be lower than that seen in the original species and ascertainment bias may occur (Ellegren *et al*., 1995; Zane *et al*., 2002). From these original 19 loci, 10 were identified as polymorphic and suitable to use for the population study. This attrition rate of 96 % can be attributed to such factors as the lack of microsatellite sequence or duplication of sequence in each clone, unsuitable microsatellite flanking sequence for primer design and, finally, PCR resulting in no amplification product or un-interpretable bands (Squirrell *et al*., 2003).

Heterozygote deficiency, expressed as significant departure from Hardy Weinberg equilibrium (HWE), can occur as a result of inbreeding, the Wahlund effect or as the effect of the presence of null alleles (Dakin and Avise, 2004). The Wahlund effect is a reduction in heterozygosity resulting from population divergence; this can arise as a result of hybridisation or unbalanced seed and pollen dispersal (Van Rossum and Triest, 2006). Both inbreeding and the Wahlund effect may affect highly fragmented *T. cordata* populations where seed dispersal is limited. However, in both of these cases heterozygote deficiency is generally seen across all loci and is not loci specific. For the ten microsatellite loci developed for *T. cordata*, HWE was not observed at all loci and this

heterozygote deficiency at some loci is, therefore, more likely to be as a result of null alleles (Dakin and Avise, 2004).

Three loci, with a mean null allele frequency of 0.32, were removed from the analysis. Although the null alleles identified in the *T. cordata* were not identified at all loci, if the *T. cordata* and *T. platyphyllos* populations were considered separately, as suggested by Moura *et al* (2013), nulls were not found to occur consistently across both groups. Null alleles were identified as possible at tc1-45, tc2-86 and tc2-07 for *T. cordata* populations and at only tc1-30 for *T. platyphyllos*, where tc2-86 was identified as monomorphic. This inconsistency across species suggests that these may not be null alleles but may possibly arise from violations of HWE. Consequently, these loci, despite having low estimated null allele frequencies of ≤ 0.1, were included in the analysis.

The microsatellite loci successfully amplified DNA from two distant populations of *T. cordata* as well as DNA from a natural ancient population of *T. platyphyllos* and an 18th century planted avenue of *T. platyphyllos* trees. Determination of genetic diversity indicators, such as Na, Ne, Ho and He, for the two populations of *T. cordata* trees and the two populations of *T. platyphyllos*, shows no significant differences between any of the populations. This is in contrast to the results obtained for the *T. platyphyllos* microsatellites designed by Phuekvilai (2014) where *T. platyphyllos* populations were significantly more diverse. The results derived from the *T. cordata* microsatellite loci used with *T. platyphyllos* trees, however, are most likely affected by the small number of samples used (Pruett and Winker, 2008).

The microsatellite loci were also found to amplify DNA in *T. platyphyllos*, as well as a very small number of *T. tormentosa and T. x euchlora* trees collected from Riseholme

Park. *Tilia x euchlora* is thought to be a hybrid between *Tilia dasystyla* Steven and possibly *T. cordata* and is tetraploid (Pigott, 2012). Although widely occurring, it is only known as a planted tree and most individuals are probably clones (Pigott, 2012). The three trees from Riseholme Park were identified as possible clones with identical genotype at all ten microsatellite loci. The three trees had, however, not been planted at the same time, with the youngest tree having a diameter at breast height of 0.3 m compared with that of the two older trees of 0.6 m. Although for *T. cordata* and *T. platyphyllos* a maximum of two alleles for each locus had been identified, for *T. x euchlora* three alleles were observed at tc2-07 and tc2-69 and four alleles at tc3-57, suggesting that *T. x euchlora* may be polyploid.

T. cordata and *T. platyphyllos* share alleles at all ten of the microsatellite loci but despite this the two species remain separate (Pigott, 2012). Shared alleles can arise from either gene flow between the two hybridising species or from variation in shared ancestral populations. Muir and Schlötterer (2005) propose that, for two hybridising oaks (*Quercus petraea* and *Quercus robur*), the sharing of alleles is a consequence of having shared ancestors. With a similar history to oak of expansion from glacial refugia, shared chloroplast haplotypes and evidence of only a limited number of hybrids within the population (< 10% of trees within the Lincolnshire Limewoods were identified as hybrids (chapter 4)) it seems possible that *Tilia spp*. also share alleles as a result of their shared ancestry.

The locus tc2-16 appears to be fixed for *T. cordata* but for *T. platyphyllos*, although three alleles were detected, only one of these was at high frequency. As tc2-16 is fixed for *T. cordata* and predominantly fixed for *T. platyphyllos*, if the incidence of introgression is

not significant, it may be possible to use this single locus to identify F1 hybrids in a population in a single PCR reaction. Further investigation should be carried using additional *T. platyphyllos,* trees of known hybrid origin and other trees from the genus *Tilia*. This could also be extended to include Asian and North American *Tilia* species and would enable the feasibility of using tc2-16 to identify hybrids and other *Tilia* to be determined.

3.5 References

Beatty, G.E., Brown, J.A., Cassidy, E.M., Finlay, C.M., McKendrick, L., Montgomery, W.I., Reid, N., Tosh, D.G. and Provan, J. (2015) Lack of genetic structure and evidence for long-distance dispersal in ash (*Fraxinus excelsior*) populations under threat from an emergent fungal pathogen: implications for restorative planting. *Tree Genetics & Genomes*, 11(3) 1-13.

Beaumont, M.A. and Bruford, M.W. (1999) Microsatellites in conservation genetics. In: D. B. Golstein and C. Schlotterer (eds) *Microsatellites Evolution and Applications.* 1st edition. Great Britain: Oxford University Press, 165-182.

Birks, H.J.B. (1989) Holocene isochrone maps and patterns of tree-spreading in the British Isles. *Journal of Biogeography*, 16(6) 503-540.

Chapuis, M. and Estoup, A. (2007) Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution*, 24(3) 621-631.

Dakin, E.E. and Avise, J.C. (2004) Microsatellite null alleles in parentage analysis. *Heredity*, 93(5) 504-509.

Duan, C.X., Li, D.D., Sun, S.L., Wang, X.M. and Zhu, Z.D. (2014) Rapid development of microsatellite markers for *Callosobruchus chinensis* using Illumina paired-end sequencing. *PloS one*, 9(5) 95458.

Ellegren H., Primmer C.R., Sheldon B. (1995) Microsatellite evolution: directionality or bias in locus selection? *Nature Genetics*, 11(4) 360-362.

Eisen, J. (1999) Mechanistic basis for microsatellite instability. In: D. B. Golstein and C. Schlotterer (eds.) *Microsatellites Evolution and Applications*. 1st edition. Great Britain: Oxford University Press, 34-48.

Filatov, D.A. (2002) ProSeq: A software for preparation and evolutionary analysis of DNA sequence data sets. *Molecular Ecology Notes*, 2(4) 621-624.

Fineschi, S., Salvini, D., Taurchini, D., Carnevale, S. and Vendramin, G.G. (2003) Chloroplast DNA variation of *Tilia cordata* (Tiliaceae). *Canadian Journal of Forestry Research*, 33 2503-2508.

Fromm, M. and Hattemer, H.H. (2003) Inheritance of allozymes and hybridization in two European *Tilia* species. *Heredity*, 91(3) 337-344. Available from http://www.proquest.com/; Document ID: 868149881 [Accessed May 31, 2011].

Glenn, T.C. and Schable, N.A. (2005) Isolating Microsatellite DNA Loci. In: Elizabeth A. Zimmer and Eric H. Roalson (eds.) *Methods in Enzymology*. San Diego CA: Academic Press, 202-222.

Hancock, J.M. (1999) Genomic context and mutational mechanisms. In: D. B. Golstein and C. Schlotterer (eds.) *Microsatellites Evolution and Applications*. 1st edition. Great Britain: Oxford University Press, 1-9.

Hosseinzadeh Colagar, A., Yusefi, M., Zarei, M. and Yousefzadeh, H. (2013) Assessment of genetic diversity of *Tilia rubra* DC. by RAPD analysis in the Hyrcanian forests, north of Iran. *Polish Journal of Ecology*, 61(2) 341-348.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P. and Drummond, A. (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28(12) 1647-1649.

Leese, F., Mayer, C. and Held, C. (2008) Isolation of microsatellites from unknown genomes using known genomes as enrichment templates. *Limnology and Oceanography: Methods*, 6(9) 412-426.

Liesebach, H. and Sinko, Z. (2008) A contribution to the systematics of the genus *Tilia* with respect to some hybrids by RAPD analysis. *Dendrobiology*, 59 13-22.

Lowe, A., Harris, S. and Ashton, P. (2004) *Ecological Genetics: Design, Analysis and Application*. Blackwell Publishing.

Martin, M., Mattioni, C., Cherubini, M., Taurchini, D. and Villani, F. (2010) Genetic diversity in European chestnut populations by means of genomic and genic microsatellite markers. *Tree Genetics and Genomes*, 6(5) 735.

Maurer, W.D. and Tabel, U. (1995) A methodical study to improve the isozyme analysis for identification of clones of *Tilia* (linden syn. lime tree). *Silva Geneticae*, 44(5-6) 351-356.

Moura, A.E., Tsingarska, E., Dąbrowski, M.J., Czarnomska, S.D., Jędrzejewska, B. and Pilot, M. (2013) Unregulated hunting and genetic recovery from a severe population decline: The cautionary case of Bulgarian wolves. *Conservation Genetics*, 1-13.

Muir, G., Lowe, A.J., Fleming, C.C. and Vogl, C. (2004) High nuclear genetic diversity, high levels of outcrossing and low differentiation among remnant populations of *Quercus petraea* at the margin of its range in Ireland. *Annals of Botany*, 93(6) 691-697.

Muir, G. and Schloetterer, C. (2005) Evidence for shared ancestral polymorphism rather than recurrent gene flow at microsatellite loci differentiating two hybridizing oaks (*Quercus* spp.). *Molecular Ecology*, 14(2) 549-561.

Mullis, K.B. and Faloona, F.A. (1987) Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods in Enzymology*, 155 335-350.

Peakall, R., Ebert, D., Cunningham, R. and Lindenmayer, D. (2006) Mark–recapture by genetic tagging reveals restricted movements by bush rats (*Rattus fuscipes*) in a fragmented landscape. Journal of Zoology, 268(2) 207-216.

Peakall, R. and Smouse, P.E. (2012) GenAlEx 6.5: Genetic analysis in excel. Population genetic software for teaching and research - an update. *Bioinformatics*, 28(19) 2537-2539.

Peterken, G.F. (1974) A method for assessing woodland flora for conservation using indicator species. *Biological Conservation*, 6(4) 239-245.

Phuekvilai, P. (2014) Relicts, refugia and reticulation: a study of population history, hybrids and phylogeny in the long-lived flowering tree genus *Tilia*. PhD Newcastle University.

Phuekvilai, P. and Wolff, K. (2013) Characterization of microsatellite loci in *Tilia Platyphyllos* (Malvaceae) and cross-amplification in related species. *Applications in Plant Sciences*, 1(4) 1200386.

Pigott, C.D. (1969) The status of *Tilia cordata* and *T. platyphyllos* on the Derbyshire limestone. *Journal of Ecology*, 57(2) 491-504.

Pigott, C.D. (1991) Biological flora of the British Isles. no 174 *Tilia cordata* Miller. *Journal of Ecology*, 79(4) 1147-1207.

Pigott, D. (2012) *Lime-trees and basswoods: a biographical monograph of the genus Tilia*. Cambridge University Press.

Provan, J., Beatty, G.E., Hunter, A.M., McDonald, R.A., McLaughlin, E., Preston, S.J. and Wilson, S. (2008) Restricted gene flow in fragmented populations of a wind-pollinated tree. *Conservation Genetics*, 9(6) 1521-1532.

Pruett, C.L. and Winker, K. (2008) The effects of sample size on population genetic diversity estimates in song sparrows *Melospiza melodia*. *Journal of Avian Biology*, 39(2) 252-256.

Raymond, M. and Rousset, F. (1995) GENEPOP (version 1.2): Population genetics software for exact tests and ecumenism. *Journal of Heredity*, 86(3) 248.

Rozen, S. and Skaletsky, H.J. (2000) Primer 3 on the WWW for general users and for biologist programmers. In: S. Krawetz and S. Misener (eds.) *Bioinfomatics Methods and Protocols; Methods in molecular Biology*. Totowa, NJ: Humana Press, 365-386.

Sambrook, J. and Russell, D.W. (2001a) *Molecular cloning: a laboratory manua*l. 3rd edition. Cold Spring Harbor, N. Y. Cold Spring Harbor Laboratory Press, 5.23-5.25.

Sambrook, J. and Russell, D.W. (2001b) Recovery of DNA from agarose and polyacrylamide gels: electroelution into dialysis bags. In: *Molecular cloning: a laboratory manual*. 3rd edition. Cold Spring Harbor, N. Y. Cold Spring Harbor Laboratory Press, 5.23-5.25.

Selkoe, K.A. and Toonen, R.J. (2006) Microsatellites for ecologists: A practical guide to using and evaluating microsatellite markers. *Ecology Letters*, 9 615-629.

Squirrell, J., Hollingsworth, P.M., Woodhead, M., Russell, J., Lowe, A.J., Gibby, M. and Powell, W. (2003) How much effort is required to isolate nuclear microsatellites from plants? *Molecular Ecology*, 12(6) 1339-1348.

Sutherland, B.G., Belaja, A., Nier, S., Cottrell, J.E., Vaughan, S.P., Hubert, J. and Russell, K. (2010) Molecular biodiversity and population structure in common ash (*Fraxinus excelsior* L.) in Britain: Implications for conservation. *Molecular Ecology*, 19(11) 2196-2211.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M. and Shipley, P. (2004) Micro-checker: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, 4(3) 535-538.

Van Rossum, F. and Triest, L. (2006) Fine-scale genetic structure of the common *Primula elatior* (Primulaceae) at an early stage of population fragmentation. *American Journal of Botany*, 93(9) 1281-1288.

Vanden Broeck, A., Storme, V., Cottrell, J.E., Boerjan, W., Van Bockstaele, E., Quataert, P. and Van Slycken, J. (2004) Gene flow between cultivated poplars and native black poplar (*Populus nigra* L.): A case study along the river Meuse on the Dutch–Belgian border. *Forest Ecology and Management*, 197(1–3) 307-310.

Vaughan, S.P., Cottrell, J.E., Moodley, D.J., Connolly, T. and Russell, K. (2007) Distribution and fine-scale spatial-genetic structure in British wild cherry (*Prunus avium* L.). Heredity, 98(5) 274-283.

Weising, K., Nybom, H., Wolff, K. and Kahl, G. (2005) *DNA fingerprinting in plants. Principles, methods and applications*. 2nd edition. USA: CRC Press.

Welsh, J. and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research*, 18(24) 7213-7218.

Yousefzadeh, H., Hosseinzadeh Colagar, A., Tabari, M., Sattarian, A. and Assadi, M. (2012) Utility of ITS region sequence and structure for molecular identification of *Tilia* species from Hyrcanian forests, Iran. *Plant Systematics & Evolution*, 298(5) 947-961.

Zane, L., Bargelloni, L. and Patarnello, T. (2002). Strategies for microsatellite isolation: a review. *Molecular Ecology*, 11(1) 1-16.

CHAPTER FOUR

The Genetic Variation and Structure of *Tilia cordata* within the Lincolnshire Limewoods.

4.1 Introduction

For plants, lifestyle factors such as range, fecundity, method of reproduction and pollination and generation time have been found to affect genetic diversity (Hamrick *et al*., 1979; Loveless and Hamrick, 1984). Large, long living tree species that have large continuous populations, outcrossing breeding systems and long distance pollen and seed dispersal are able to maintain high levels of genetic diversity (Hamrick *et al*., 1992), whereas small, isolated and fragmented populations are potentially at risk of experiencing a reduction in genetic diversity as a consequence of increased levels of inbreeding and genetic drift (Couvet, 2002; Ellstrand and Elam, 1993). However, even in situations where plant populations are highly fragmented with small numbers of individuals (<1500) some plants, such as *Argyroxiphium kauense* (Rock and Neal) Degener and Degener (Friar *et al*., 2001) and *Rhododendron protistum* var. *giganteum* L. (Wu *et al*., 2014), appear to be able to maintain high levels of genetic diversity within their populations. With other plants where populations are considered critically endangered, such as *Breonnadia salicina* (Vahl) Hepper and J.R.I. Wood (Gaafar *et al*., 2014) in Saudi Arabia and *Saxifaga hirculus* L.(Beatty *et al*., 2014) in Ireland, the loss of genetic diversity is a major concern in the development of a conservation strategy.

Hybridisation occurs when plants breed with other species or with members of the same species that are genetically divergent. This process results in allele sharing and can increase the species diversity (Soltis and Soltis, 2009). Hybridisation is an important factor in plant evolution and, although it can result in the extinction of species when rare and abundant species meet (Rhymer and Simberloff, 1996), natural hybridisation is generally considered to increase diversity with many plant species originating from hybridising events (Soltis and Soltis, 2009).

Even though woodlands are often genetically uniform with genetic variation being mainly distributed within populations (Hamrick *et al*., 1992), fine scale spatial genetic structure (SGS) can occur within woodlands. SGS arises from the interaction between gene dispersal, genetic drift, natural selection and the spatial distribution of individuals (Loveless and Hamrick, 1984; Epperson, 1995) and can provide some insight into the processes that are needed to conserve viable populations (Escudero *et al*., 2003). For plants, SGS arises predominantly from the interaction between local genetic drift and seed and pollen dispersal (Heywood, 1991). When neighbours are more similar to each other than to distant individuals, fine scale genetic structure increases. Limited pollen and seed dispersal and practices such as self-fertilisation all increase the fine scale genetic structure. In a study by Cottrell *et al*. (2003), *Q. petraea* was identified with a greater level of SGS than Q. *robur*. This was attributed to differences in seed dispersal strategies for the two species, with *Q. robur* seed being more likely to be dispersed over long distances by birds. In *Primula elatior* Hill significant SGS was attributed to restricted pollen and seed dispersal (Van Rossum and Triest, 2006). SGS is useful in helping to determine how genetic interactions can be influenced by a species' life history characteristics (Degen *et al*., 2001).

Microsatellites, or Simple Sequence Repeats (SSR), are one of several molecular markers available for population genetics study (Lowe *et al*., 2004). They are, however, the molecular marker of choice for this study to investigate the inter- and intra-population genetic diversity of *T. cordata*. Molecular markers based on chloroplasts, with their lower mutation rates and lack of recombination are suitable for broad studies across taxonomic groups and can inform about phylogeographic changes over long distances and time (Provan *et al*., 2001). However, microsatellites are more able to distinguish between

individuals and provide insight into more recent events and are suitable for investigations into ecological questions on a local level (Selkoe and Toonen, 2006).

Tilia cordata Mill. and *Tilia platyphyllos* Scop. are members of the Malvaceae family in the order of Malvales. They are in a group of four trees that are native to Europe and are distributed throughout the region, with *T. cordata* being the more widespread and found at more northern latitudes than *T. platyphyllos* (Svejgaard Jensen, 2003) (figure 1.4). The trees are generally insect pollinated and when they occur together the two trees can hybridise and produce fertile offspring. This is normally prevented from occurring as *T. platyphyllos* flowers about ten days before *T. cordata* (Pigott, 2012). Pigott (1969) has documented the occurrence of *T. cordata*, *T. platyphyllos* and hybrid trees within native Limewoods in Derbyshire and Logan *et al*. (2015) has investigated their incidence in a number of other British woods, identifying 6.1% of the trees sampled as hybrids or introgressed individuals.

Pollen records (Birks, 1989) show that after the last ice age both *T. cordata* and *T. platyphyllos* arrived in Britain after expansion from glacial refugia in Europe about 8,000 BP. The identification of glacial refugia, by detecting areas with high levels of genetic diversity, and the most likely routes for the re-colonisation of Europe after the last episode of glaciation, has been determined for many species (Hewitt, 1999; Provan and Bennett, 2008). Populations expanding from refugia at the end of the glacial period have been shown to experience isolation by distance and an increase in homozygosity as a consequence of leading edge colonisation during the postglacial expansion (Hewitt, 1999). Diversity within chloroplast haplotypes has been used to support the proposal that the Iberian Peninsular was the most likely glacial refugium for British *Quercus petraea*

(Matt.) Liebl. and *Quercus robur* L. (Cottrell *et al*., 2002) and that *T. cordata* possibly colonised Britain from refugia in Italy, the Balkans and Iberia, while *T. platyphyllos* may have its post glacial origins within the Iberian Peninsular (Phuekvilai 2014).

The Lincolnshire Limewoods are Ancient Semi-Natural Woodlands (ASNW) within central Lincolnshire. Although these woods have been managed as an important resource for local communities over many hundreds of years, the biodiversity contained within them suggests that they are very old and are possibly remnants of forests that covered Britain after the last ice age (Pigott and Huntley, 1980; Rackham, 2008). Today they exist as isolated woods separated from each other by tracts of intensively farmed land. The investigation into the genetic diversity using microsatellites will enable genetic differences between the woods to be identified. As lower levels of genetic diversity can be associated with the loss of population viability and evolutionary potential (Young *et al*., 1996), this study aims to determine whether the history and management of the *T. cordata* trees within the Lincolnshire Limewoods has resulted in them becoming differentiated, with lower genetic diversity than *T. cordata* in populations outside Lincolnshire. Within the Lincolnshire Limewoods themselves, fine scale genetic structure, the identification of large clonal groups and determining the incidence of *T. platyphyllos* and the presence of hybrids will all help to inform conservation strategies for the woods and will help to ensure that the genetic diversity can be conserved within the populations.

4.2 Materials and Methods

4.2.1 Sample collection, DNA extraction and PCR

Tilia cordata samples were collected from the Lincolnshire Limewoods for both the population genetic studies and the tissue culture studies of this work. The trees sampled were located either in the Bardney Limewoods or were from two additional Lincolnshire woods outside the area; Skellingthorpe Old Wood and Potterhanworth Wood. Potterhanworth Wood is separated from the Bardney Limewoods by the River Witham and lies 10 km to the south west of the Chambers Farm group of woods; Skellingthorpe Old Wood lies 25 km to the west. The trees used for the study were mapped using GPS (Garmin Gekko 201) with at least 20 trees being selected from each wood. The sampling of the trees was not statistically random but haphazard with the distance between the trees and their location depending on accessibility and density of lime trees within the wood (Lowe *et al*., 2004). In general, however, the sampling distance between adjacent trees was > 30 m (mean 62.58m, s.d. 40.2m).

Tree samples, stems 30 to 40 cm in length with 6 to 10 buds, were collected in the spring, before the leaves appeared and the woods were easier to access. The samples were kept in water in clean laboratory conditions and the leaves were allowed to emerge (figure 4.1). Leaf tissue and buds collected this way were used for both tissue culture and DNA extraction. The tissue required for DNA extraction was either taken directly from a fresh leaf sample or from samples stored at -70 ºC.

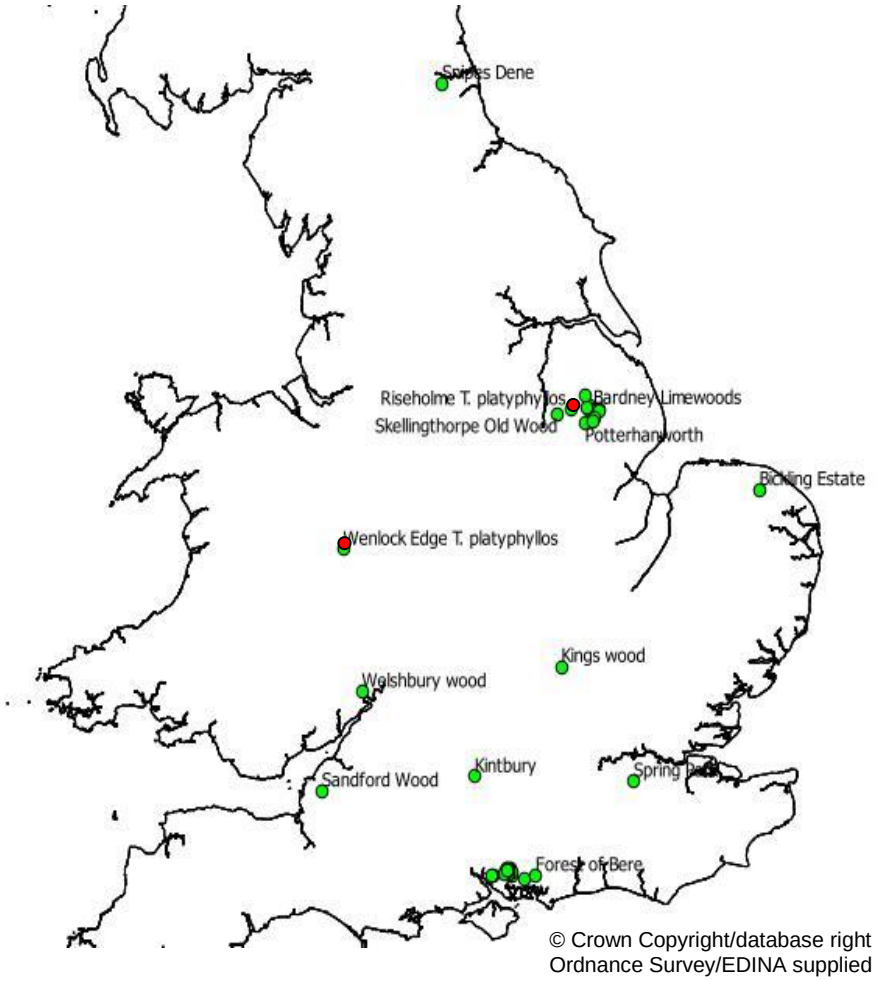
For comparison with the Lincolnshire Limewoods populations, additional samples for analysis were collected by members of the Limewoods Working Group, from ASNW

woods throughout England (figure 4.2). Young leaves were collected and individual samples stored in paper envelopes. To desiccate and preserve the leaves for DNA analysis the samples were placed in sealed plastic bags containing silica gel. To ensure that the leaves completely dried and did not deteriorate, the silica gel was replaced and regenerated at regular intervals until the gel remained dry. Samples collected this way were stored with silica gel at room temperature (Chase and Hills, 1991).

A total of 469 *Tilia* spp. trees were sampled from 43 woods in England (figure 4.2, table 4.1. More detailed maps with individual tree location are given in appendix 1). These include two populations of known *T. platyphyllos*, a "wild" population from Wenlock Edge, Shropshire (number of trees sampled N = 7) and a planted population from Riseholme Park, Lincolnshire (N = 8). Putative *T. cordata* trees (N = 274) were sampled from 14 woods from the Bardney Limewoods with a further 39 trees being sampled from two additional Lincolnshire woods to the west of the Limewoods area. For comparison with these, 141 trees were collected from 25 woodland areas outside Lincolnshire, 17 of these woods being within the Forest of Bere, Hampshire. The number of trees used in the final data sets was reduced by the removal of trees with more than 20% missing genotype data and those which were identified as clones. Trees in close proximity which had identical genotypes were identified as clones using GenAlEx 6.501 (Peakall and Smouse, 2006; Peakall and Smouse, 2012) and GenClone v2 (Arnaud-Haond and Belkhir, 2007).

Figure 4.1 *T. cordata* samples with newly emerged leaves.

Figure 4.2 Location of woods and woodland areas in England where *Tilia* spp. samples were collected (Ordnance Survey, 2014a).



- Wood location
- *T. platyphyllos* populations

Table 4.1 Location of the woods from which *Tilia cordata* Mill were sampled, using decimal latitude and longitude coordinates. The wood group and the regional identity (R) of the woods are also indicated.

Wood name		Wood group	R	County	No. trees collected	Latitude	Longitude
Great Scrubbs	*	Bardney L'woods	L	Lincolnshire	25	53.25357	-0.27876
Hatton Wood	*	Bardney L'woods	L	Lincolnshire	18	53.25993	-0.25726
Ivy Wood	*	Bardney L'woods	L	Lincolnshire	31	53.24873	-0.28543
Little Scrubbs Wood	*	Bardney L'woods	L	Lincolnshire	20	53.25407	-0.28808
Minting Wood		Bardney L'woods	L	Lincolnshire	6	53.25279	-0.26011
Cocklode Wood	*	Bardney L'woods	L	Lincolnshire	30	53.27606	-0.34617
College Wood	*	Bardney L'woods	L	Lincolnshire	20	53.26477	-0.31374
Goslings Corner Wood	*	Bardney L'woods	L	Lincolnshire	20	53.26273	-0.29019
Hardy Gang Wood	*	Bardney L'woods	L	Lincolnshire	20	53.25913	-0.35774
Newball Wood	*	Bardney L'woods	L	Lincolnshire	20	53.27048	-0.36716
Rand Wood		Bardney L'woods	L	Lincolnshire	5	53.28903	-0.38030
Scotgrove Wood	*	Bardney L'woods	L	Lincolnshire	20	53.21993	-0.30792
Southrey Wood	*	Bardney L'woods	L	Lincolnshire	20	53.19956	-0.31349
Wickenby Wood	*	Bardney L'woods	L	Lincolnshire	21	53.33368	-0.38096
Potterhanworth Wood	*		L	Lincolnshire	20	53.18877	-0.39523
Skellingthorpe Old Wood	*		L	Lincolnshire	19	53.23772	-0.65162
Bottom Copse		Forest of Bere	S	Hampshire	2	50.8959	-1.29454
Vantage Copse		Forest of Bere	S	Hampshire	5	50.89898	-1.29791
Abbots Hill Wood	*†	Forest of Bere	S	Hampshire	3	50.90518	-1.13668
Bere Copse		Forest of Bere	S	Hampshire	2	50.93039	-1.14048
Bishops Wood		Forest of Bere	S	Hampshire	1	50.92108	-1.17138
Gardiners Purlieu		Forest of Bere	S	Hampshire	2	50.90432	-1.14238
Goat House Wood		Forest of Bere	S	Hampshire	2	50.8971	-1.13825
Hipley Copse		Forest of Bere	S	Hampshire	1	50.8966	-1.11963
Huntbourne Wood		Forest of Bere	S	Hampshire	7	50.91224	-1.11806
Lymington Purlieu	*	Forest of Bere	S	Hampshire	7	50.91243	-1.14366
Mill Copse		Forest of Bere	S	Hampshire	2	50.92683	-1.14624
West Walk	*†	Forest of Bere	S	Hampshire	4	50.91251	-1.15503
W. Wickham relic tree		Forest of Bere	S	Hampshire	1	50.90821	-1.19650
Coach Road Copse		Forest of Bere	S	Hampshire	1	50.93251	-1.16051
Great Lion Copse	*	Forest of Bere	S	Hampshire	10	50.9308	-1.16011
Huntage Copse	*	Forest of Bere	S	Hampshire	10	50.9248	-1.16292
Hurst Wood		Forest of Bere	S	Hampshire	5	50.88173	-1.01486
Stanstead Wood			S	Hampshire	2	50.89614	-0.91502
Bickling Estate				Norfolk	4	52.81962	1.20860
Kintbury			C	Berkshire	8	51.39585	-1.373250
Kings Wood			C	Buckinghamshire	6	51.95699	-0.65481
Spring Park	*		C	GLC	17	51.36293	-0.01264
Sandford Wood	*		W	Somerset	17	51.32827	-2.82112
Welshbury Wood	*		W	Gloucestershire	18	51.83602	-2.46777
Snipes Dene				Tyne and Wear	11	54.92896	-1.71909
Wenlock Edge		*T. platyphyllos*		Shropshire	7	52.56552	-2.63728

* Woods with ten or more trees sampled, used for population study.

† Adjacent wood, samples amalgamated with Lymington Purlieu to form >10 tree group.

R Region: L - Lincolnshire, S - South, C - Central, W - West

DNA was extracted from single *Tilia* leaves using a modified CTAB extraction protocol (chapter 2.1). Microsatellite markers were developed for *T. cordata* using the method devised by Glenn and Schable (2005) (chapter 3) The resulting ten microsatellite primer pairs, labelled with either 6FAM, HEX (Sigma Aldrich) or NED (Applied Biosystems) to facilitate multiplex PCR, were used to amplify the genomic DNA. For each 6.0µl multiplex reaction 3.0µl 2xQuiagen PCR Multiplex Master Mix, 0.6µl 10x primer mix (to give a final concentration of each primer of 0.2µM), 1.2µl ddH_2O, 1.2µl DNA template (DNA≈ 1ng/µl) was used. The thermocycling reaction conditions applied were an initial activation step of 95 ºC for 15mins followed by 35 cycles of 94 ºC for 30s, the appropriate primer anneal temperature (table 3.2) for 90s and 72 ºC for 60s, followed by a final extension of 60 ºC for 30mins. Fragments, together with GeneScan™ 350 ROX™ internal size standard, were run on an ABI3730 automated DNA analyser. A separate allelic ladder, derived from known fragments from selected trees was included on each PCR plate to enable consistent fragment size scoring across all the fragment plates. Electropherograms were analysed using Geneious v 6.1.2 (http://www.geneious.com, (Kearse *et al*., 2012)). The null allele frequency was investigated with Micro-Checker (Van Oosterhout *et al*., 2004).

4.2.2 Identification of Clonal Ramets

To identify clonal ramets, the microsatellite data from the complete data set of trees (N = 443) was analysed to identify trees with identical sequences across all of the 10 loci. Trees identified in this way, that also occurred in close geographical proximity were identified as ramets of genet. The chance that two unrelated randomly mating trees have the same genotype can be estimated by calculating the probability of identity (PI), where $PI = 2 (\Sigma p_i^2)^2 - \Sigma p_i^4$ with p_i the frequency of the ith allele at a locus and PI for multiple loci being the product of the PI's from individual loci (Peakall *et al*., 2006; Wilk *et al*., 2009). Peakall *et al*. (2006) also suggests that the reciprocal of the population size gives a value for PI below which there is an expectation that trees with matching genotypes are unlikely to be identical by chance and are more likely to be clones of the same individual (Peakall *et al*., 2006; Wilk *et al*., 2009). If members of the population are likely to be related then the calculation of the probability of identity of siblings (PIsibs) gives a more conservative result for the probability of identity. Identification of identical genotypes, trees that differed at only one allele, and the estimation of the probability of identity was carried out using GenAlEx 6.501 (Peakall *et al*., 2006; Peakall and Smouse, 2012) and GenClone v2 (Arnaud-Haond and Belkhir, 2007) (downloaded from http://www.ccmar.ualg.pt/maree/software.php?soft=genclon)

4.2.3 Identification of *T. platyphyllos* and Occurrence of Hybridisation within the Population Groups

The incidence of hybridisation and the occurrence of *T. platyphyllos* within the sampled populations were investigated with NewHybrids (Anderson and Thompson, 2002) (downloaded from http://ib.berkeley.edu/labs/slatkin/eriq/software/software.htm). This uses a Bayesian approach to identify the posterior distribution of hybrids occurring in the population from the genotype data. Knowledge of the allele frequencies of the parent species is not required and the populations are assumed to be in Hardy Weinberg and linkage equilibrium. The predictable pattern of inheritance of genes in hybrids is used to assign individuals to a genotype class. For the analysis of *Tilia* spp. the data was assigned to one of six genotype frequency classes, pure *T. cordata*, pure *T. platyphyllos*, F1 hybrid, F2 hybrid, F1 backcrossed to *T. cordata* and F1 backcrossed to *T. platyphyllos*. The hybrid classes into which the individual trees were expected to fall were defined in terms of the expected proportion of loci arising from either *T. cordata* or *T. platyphyllos* or from hybrids derived from both species and were defined in the genotype frequency class input file (table 4.2). Only level one back crosses were considered in the analysis as the probability of miss-identifying more advanced back crosses as their parents increases unless the number of loci analysed is increased (Boecklen and Howard, 1997).

The genotype data from all trees sampled was used (the total number of trees when duplicated clones have been excluded, N = 453). The programme was run with no prior information about the hybridisation state of any of the trees. The Markov Chain Monte Carlo simulation (MCMC) was reset after a burn-in of period of 3×10^6 sweeps and run for 10^7 sweeps. The simulation was repeated once from a different random number start. Jeffreys-like priors were used for the analysis as the effects of using either Jeffreys-like or

uniform priors for π, the mixing proportions, and θ, the allele frequencies, were found not to alter the results.

Table 4.2 Genotype input data for NewHybrids with the expected genotype frequency of the hybrid classes from the association between the two populations *T. cordata* (1) and *T. platyphyllos* (0).

Genotypes	0 x 0	0 x 1	1 x 0	1 x 1
Hybrid class				
Pure 0	1	0	0	0
Pure 1	0	0	0	1
F1	0	0.5	0.5	0
F2	0.25	0.25	0.25	0.25
0 Bx	0.5	0.25	0.25	0
1 Bx	0	0.25	0.25	0.5

T. cordata = Pure 1
T. platyphyllos = Pure 0
F1 = Pure 0 x pure1
F2 = F1 x F1
0 Bx = F1 x 0 (Backcross)
1 Bx = F1 x 1 (Backcross)

4.2.4 Identification of Population Genetic Diversity

To describe the population genetic diversity within the woodlands and estimate the impact of the occurrence of hybrid trees and *T. platyphyllos* on the genetic diversity, trees were analysed in population groups (table 4.1), using data from all the trees sampled. (*T. cordata* as well as *T. platyphyllos* and *platyphyllos / cordata* hybrids (all *Tilia*)), and compared with the same populations with only the putative *T. cordata* included (*T. cordata*). *T. platyphyllos* and *platyphyllos / cordata* hybrids were identified both morphologically (table 1.2) and from the results obtained using NewHybrids (Anderson and Thompson, 2002) (this section 4.3.2). Woods with ten or more trees, i.e. 20 woods from the total collection, were used to assess the genetic diversity of the populations. Woods with small numbers of trees (less than ten) were, if possible, combined with suitable adjoining woods to make larger populations or otherwise excluded from this part of the study. Genetic diversity was also compared within larger regional population groups made up from trees from the south (including the Forest of Bere), the west (Welshbury and Sandford Woods), central (Kings Wood, and Spring Park) and all the Lincolnshire Woods. A single population (number of trees N = 453) of all the trees sampled was used for the investigations into the population structure using factorial correspondence analysis and Bayesian cluster analysis.

The populations were tested for deviations from Hardy Weinberg equilibrium via Fisher's exact test and for linkage disequilibrium using GENEPOP (Raymond and Rousset, 1995; Rousset, 2008). Weir and Cockerham's (1984) estimate of the inbreeding coefficient, F_{IS}, was determined and exact P-values were estimated using the Markov Chain method with 10^4 dememorizations and 10^5 iterations.

Overall genetic diversity was estimated for each of the sampled populations or regions using GenAlEx 6.501 (Peakall *et al*., 2006; Peakall and Smouse, 2012). Values for the number of alleles (Na), number of private alleles (Np), effective number of alleles (Ne), level of observed heterozygosity (Ho) and expected heterozygosity (He) and fixation index (F) were calculated for all loci and mean values were determined for all populations. The significance of F was derived using FSTAT 2.9.3.2 (Goudet, 1995; Goudet, 2001) based on 10^4 randomisations. The effect on genetic diversity of the spread of *T. cordata* northwards towards the edge of its geographic range was considered by determining the correlation between the latitude of *T. cordata* populations and the expected heterozygosity (He). To assess the differences in genetic diversity between the populations with and without the *T. platyphyllos* and hybrid populations, statistical analysis of the results was carried out using paired t-tests (using StatPac for Windows 15.1.16 (available from www.statpac.com)).

4.2.5 Population Structure

Analysis of molecular of variance (AMOVA) (Excoffier *et al*., 1992) was used to determine how genetic diversity was partitioned among and between the wood groups. AMOVA was implemented using GenAlEx 6.501(Peakall *et al*., 2006; Peakall and Smouse, 2012) with 10^4 iterations; statistical testing was based on the comparison of the results with those obtained from random permutations. Pairwise F_{ST} between wood groups, estimated via AMOVA, were visualised using principal coordinate analysis (PCA) to investigate genetic relationships among the 21 wood groups. Isolation by distance (IBD) of all the wood groups was also considered using a Mantel test (Mantel, 1967; Smouse *et al*., 1986) to compare genetic distance, in this case pairwise F_{ST}, and geographic distance matrices. PCA and Mantel tests were implemented using GenAlEx

6.501 (Peakall *et al*., 2006; Peakall and Smouse, 2012) with 10^4 iterations. Statistical testing for Mantel was based on comparison of the results with those obtained from random permutations.

Factorial Correspondence Analysis (FCA) was used to investigate the population structure of the trees. FCA is a multidimensional analytical technique that can be used to explore and visualise the relationships between individuals when there is little or no prior information about the population structure. Data points that occur close together have similar genotype profiles whereas those occurring further apart are more genetically distinct. The first and second axes of the plots explain most of the genetic variance that occurs within the data with subsequent axes accounting for less variance. Factorial correspondence analysis was carried out using GENETIX v. 4.05 (Belkhir *et al*., 1996-2004)

The population structure of *Tilia* spp. (N = 453) was further examined using the Bayesian analysis program STRUCTURE 2.3.4 (Pritchard *et al*., 2000; Falush *et al*., 2003; Harter *et al*., 2004; Hubisz *et al*., 2009). STRUCTURE is a model-based software that uses a Bayesian clustering approach to infer population structure from genotype data. The posterior probability that individuals belong to a particular cluster or category is computed using a Gibbs sampling Markov Chain Monte Carlo (MCMC) algorithm. STRUCTURE can assign individuals to a number of cryptic population groups (K) without influence from any prior population information such as geographical location and, therefore, can give an indication of metapopulation structure. The individuals are assigned to clusters based on Hardy Weinberg and linkage equilibrium groups. In the

admixture model for a given number of clusters (K) the program assigns, to each individual, proportions of membership of each cluster according to genotype.

For the analysis the ancestry admixture model and the correlated allele frequency models were used in the simulation. The ancestry admixture model is a simulation whereby each individual is assumed to derive a proportion of its genetic variation from each of the K populations; this is more similar to real data and allows hybridisation to be considered. The correlated allele frequency model facilitates accurate population assignment for closely related population groups that are likely to have similar allele frequencies. Changing the value of K allows different population models to be compared and the most likely number of clusters that best explain the population structure of the complete dataset to be chosen. Pritchard *et al*. (2000) suggests using the maximum posterior probability of K estimated from the value of ln Pr (X │K) from each simulation to give an "*ad hoc* guide" to the most likely value for K. The value of K can also be estimated using the method devised by Evanno *et al*. (2005). Both methods, however, can only give a guide to the true value of K and any value of K chosen needs to be selected to represent the population in a biologically sensible way and reflect all the information available from the STRUCTURE programme.

The data, with no prior population information about the samples, was used in the computer simulation to derive an estimation of the most likely number of cryptic population clusters for the population. Exploratory tests using a burn-in of 10^5 cycles followed by 10^5 MCMC repetitions, with K values from 1 to 8, show α (the degree of admixture), F_{ST} and the likelihood (L(K)) that a particular genotype originated from a given population, all appeared to have reached equilibrium within 10^5 MCMC repetitions.

Observation of Q, the estimated membership coefficients of individuals to each cluster, also indicates that the number of populations is likely to be less than six.

STRUCTURE was configured to run with 10^6 MCMC repetitions after a burn-in of 10^5 cycles for values of K from 1 to 6. Each simulation of K was replicated 10 times to verify the results. For each value of K the data obtained over the 10 repeats was aggregated using CLUMPP1.1.2 (Jakobsson and Rosenberg, 2007). To find the optimum alignment of the replicates the full search algorithm was used for K = 3 and the algorithm GREEDY was used for values of K from 4 to 10. From the initial analysis for each value of K, the data set for all individual trees was also sorted by Q to show possible cryptic population groups that occur within the total population but are not restricted to individual woods. To identify possible structure within these sorted subpopulations, each group was reanalysed independently using K = 2 or K = 3. This was repeated, with each of the new clusters being further subdivided, until the number of populations was, if possible, reduced to one.

An estimate of the most likely number of population clusters for the group of trees was made using both Pritchard *et al*'s (2000) suggestion of using the maximum posterior probability of K based on Ln P(K) and the method devised by Evanno *et al* (2005) using ΔK, the rate of change in Ln P(K), occurring between consecutive K values.

4.2.5.1 Fine scale spatial genetic structure within local populations

Although a Mantel test was used to investigate isolation by distance (IBD) across all the trees sampled in England, fine scale spatial genetic structure (SGS) was investigated for a small group of closely situated woods (Chambers Farm Wood group, N = 68) within the Lincolnshire Limewoods, where distances between the trees was known. Multivariate spatial autocorrelation using a multi locus approach devised by Smouse and Peakall (1999) (Peakall *et al*., 2003; Double *et al*., 2005; Peakall and Smouse, 2006) was implemented with GenAlEx 6.501 (Peakall and Smouse, 2006; 2012). Genetic distance and geographic matrices were used to calculate the auto correlation coefficient r, which is closely related to Moran's I, together with 95% confidence limits about the null hypothesis and 95% confidence error bars determined by bootstrapping over 10^4 replications. Within the population, spatial autocorrelation was determined as a function of distance between the trees. For the analysis, a distance class of 50m was chosen to obtain at least 30 pairwise tree distances in each class.

The Chambers Farm wood group (Great Scrubbs, Little Scrubbs, Hatton, Minting and Ivy Woods) extends over a distance of approximately 3.4 km by 2 km and contains three SSSI woods, Hatton wood (covering an area of 36.93 ha), Little Scrubbs (13.8 ha) and Ivy Wood (15.2 ha) (Maps appendix 1). Trees within the woods were analysed as a data set containing either just *T. cordata* trees or as a group containing all the trees sampled (all *Tilia*).

4.3 Results

4.3.1 Identification of Clonal Ramets

The trees with alleles that were identical over all 10 microsatellite loci and that occurred in close proximity were considered to be ramets of clonal populations occurring as a result of vegetative reproduction (table 4 3). To assess the probability that trees would not be identified as clones by chance, the probability of identity (PI) and the probability of identity of siblings (PIsibs) were calculated for increasing numbers of microsatellite loci. For a population size of 443 the number of loci needed for a PI < 0.0023 (ie 1/443) was four and for the more conservative PIsibs to also be < 0.0023 ten loci were required (figure 4.3). With ten loci, therefore, even closely related trees are unlikely to be identical by chance. Analysis of all trees identified 12 groups of trees with identical genotypes. Trees with genotypes that varied by just one repeat at one allele were also classified as clones and a further three groups of trees were identified in this way (table 4.3).

In the Lincolnshire Limewoods and Welshbury Wood, where the location of individual trees was known, the average distance between the trees within a genet was 10.2 m (s.d. 4.6). This was much less than the average distance of 62.6 m (s.d. 40.2) between sampled trees in these woods and consequently, with limited sampling, the frequency or size of clonal groups could not be estimated in this study. Within the Forest of Bere the mean sampling distance between adjacent trees was 25 m (s.d.13.5, range 140 m to 2 m) but, even for the trees sampled at distances less than 10 m, no clones were identified. Two groups of trees from Spring Park and one group from Snipes Dene Wood had been identified on collection as possible clones and this was confirmed by the microsatellite analysis. Clonal groups were also identified among the samples collected from Kintbury

and the *T. platyphyllos* from Riseholme Park; in both of these cases the trees had been planted and had most likely used propagated material from layers or cuttings.

Duplicates of trees with identical genotypes were removed from the data set before further analysis so as to avoid bias in estimates of genetic variability and population structure (Balloux *et al*., 2003; Pilot *et al*., 2014). Other trees with missing data at one allele may also have been members of clonal groups but could not be assigned as such by either GenAlEx, where for 9 microsatellite loci PIsibs > 0.0023 and was therefore not significant for a total population of 443 trees, or GenClone ($P>0.05$) because of insufficient resolution power of the microsatellites. Data from these trees were, however, included in the study and may affect the determination of population diversity and structure.

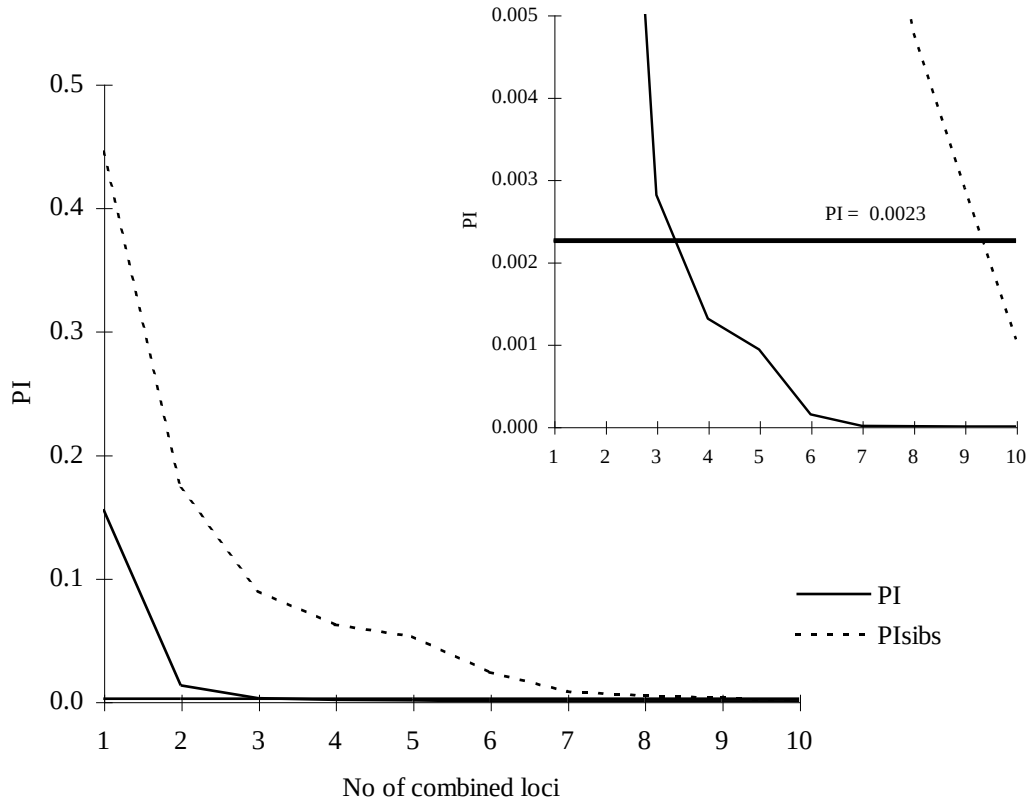
Table 4.3. The identification of trees with identical allelic structure and trees with alleles that differ by one SSR at one allele.

Wood	Identical genotype	1 SSR difference at 1 allele	Mean distance apart (m)	Latitude	Longitude
Great Scrubbs Wood	ChambL2		10.3	53.25006	-0.2801901
	ChambL1			53.25002	-0.2803267
	Chamb1A		7.3	53.25366	-0.2784568
	Chamb2			53.25372	-0.2784244
		Chamb(8)	18.4	53.25360	-0.27875899
		Chamb(7)		53.25359	-0.27875935
		Chamb5		53.25383	-0.27865972
Ivy Wood	Ivy10		11.7	53.24866	-0.2854472
	Ivy1a			53.24869	-0.2852810
Welshbury Wood	W6/SO/375		5.0	51.83605	-2.4681191
	W7/SO/380			51.83610	-2.4681196
	W15/SO/474		8.5	51.83694	-2.4684477
	W14/SO/455			51.83677	-2.4684023
	W13/SO/466			51.83687	-2.4684179
	W12/SO/463			51.83684	-2.4683886
Sandford Wood	Sford3/109			Wood location only	
	Sford6/112			51.32827	-2.8211225
	Sford5/111				
	Sford4/110				
		Sford13/119			
		Sford15/121			
Snipes Dene Wood	NZ2a/302*			Wood location only	
	NZ2c/94/81			54.92896	-1.7190850
	NZ2b/94/80				
	NZ2d/94/82				
	NZ96/89				
	NZ6/96/87				
Spring Park	Sppk93/3a*			Wood location only	
	Sppk92/3			51.36429	-0.0203260
	Sppk101/A*				
	Sppk96/A				
Kintbury	KintG/JD/7			Location only	
(Planted avenue)	KintF/JD/6			51.40344	-1.4493562
	KintE/JD/5				
	KintD/JD/4				
Riseholme Park	Rhtp1		38.0**	53.26605	-0.5289795
T. platyphyllos	Rhtp2			53.26587	-0.5284911
(Planted avenue)		Rhtp4		53.26519	-0.5282894
		Rhtp9			

* Trees identified by the collector as possible clones

** *T. platyphyllos* planted avenue tree distance

Figure 4.3 For the data set of 443 trees the probability of identity, PI and PIsibs for increasing numbers of combined loci. The inset graph has an expanded section of the main graph with values of PI below 0.005 and arbitrary estimate of PI = 1/443 below which trees are unlikely to be related by chance.



4.3.2 Identification of *T. platyphyllos* and Hybridisation within the Populations

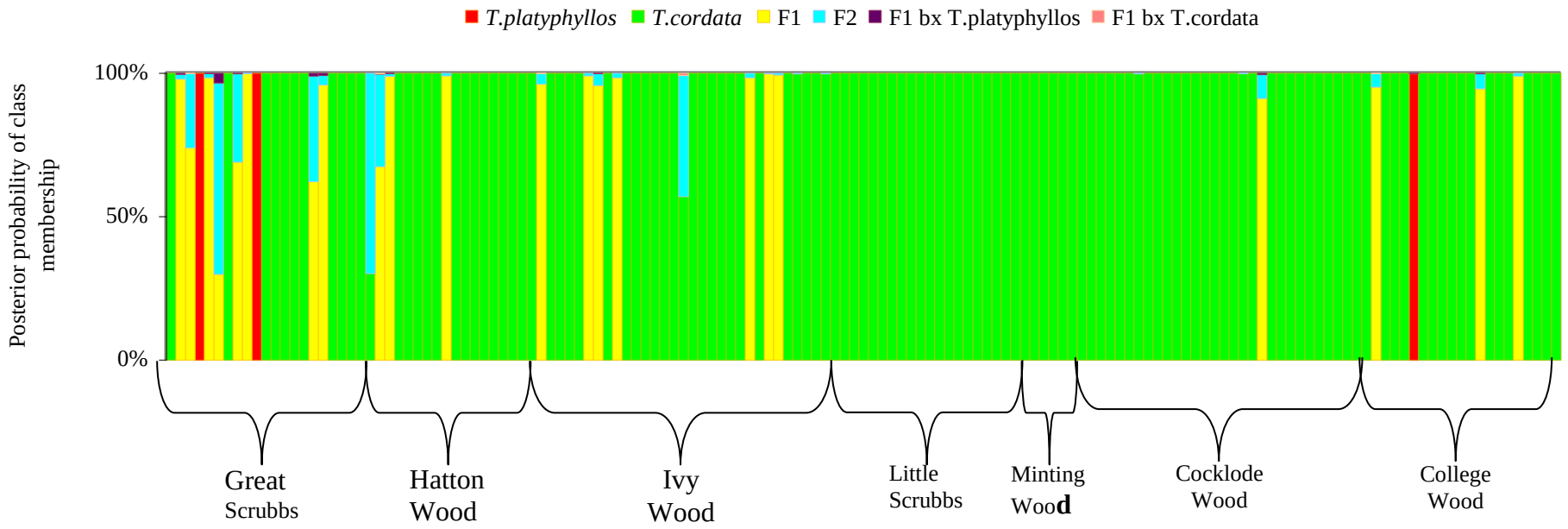
NewHybrids was configured to divide the *Tilia* spp. into six population classes and the majority of the trees collected were classed as *T. cordata*. The assignment of *T. platyphyllos* corresponded with all the trees that were known to be *T. platyphyllos*, i.e. the Riseholme Park (N = 6) and Wenlock Edge (N = 6) populations, but was also found to identify trees collected from the Lincolnshire woods that had been characterised, on collection, as having morphological features that might suggest they were not pure *T. cordata*. Of the 453 trees analysed 401 were classed as *T. cordata*, 19 as *T. platyphyllos* and the remaining 33 trees were classed as either F1 hybrids (29 trees) or F2 hybrids (four trees). No trees were identified as backcrosses from F1 hybrids to either of the pure species (figures 4.4 and 4.5).

Within the 20 woods used to calculate the genetic diversity, 32 trees were identified in 11 of the woods that could not be considered pure *T. cordata* (appendix 1 shows detailed maps of tree locations and tree identification). Of these 11 woods, 10 were within Lincolnshire, the largest concentration being found in the samples obtained from Great Scrubbs Wood (22 trees analysed, two *T. platyphyllos*, seven F1 hybrids and two F2 hybrids), Hatton Wood (17 trees, three F1 hybrids), Ivy Wood (32 trees, seven F1 hybrids) and College Wood (20 trees, one *T. platyphyllos*, three F1 hybrids). These four woods are all within the Bardney Limewoods, with Great Scrubbs, Hatton and Ivy Wood also being members of the Chambers Farm Wood group. Within the Bardney Limewoods *T. cordata* was found exclusively in Little Scrubbs, Newball and Wickenby Wood. As well as those in Great Scrubbs Wood and College Wood, *T. platyphyllos* was identified in Southrey Wood and Potterhanworth Wood but in both cases only a single tree was identified, with no F1 hybrids. Three F1 hybrids were also found in Skellingthorpe Old

Wood but no *T. platyphyllos* were identified, even though previous surveys had indicated their presence (Woodlands Trust, 2003). The trees sampled from ancient woods outside Lincolnshire were exclusively *T. cordata* except for one F2 hybrid tree that was identified in West Walk, from the Forest of Bere. A small group of eight planted trees from Baron Court near Kintbury did not contain any pure *T. cordata* but three F1 hybrids and one F2 hybrid were identified.

The percentage posterior probability of assignment of trees to the class membership was 95% or more for all but three *T. cordata* trees and for all 19 *T. platyphyllos* trees. For F1 hybrids there was more variation in results; 15 were assigned with a percentage probability of > 97%, ten between 97 and 91% and five between 75 and 69%. Of the four F2 hybrids, two trees, West Walk 4 and Kintbury C, had > 98% probability of identity while the other two trees, CW6 and Ch001, from Great Scrubbs Wood were assigned with less confidence, 70 to 66%.

Figure 4.4 NewHybrids assignment of complete data set of all trees to the population classes: pure *T. cordata*, pure *T. platyphyllos* or to hybrid classes F1 hybrid, F2 hybrid, F1 backcrossed to *T. cordata* or F1 backcrossed to *T. platyphyllos* by posterior probability of class membership. Each bar represents one individual tree and the trees are arranged in their woods (continued).



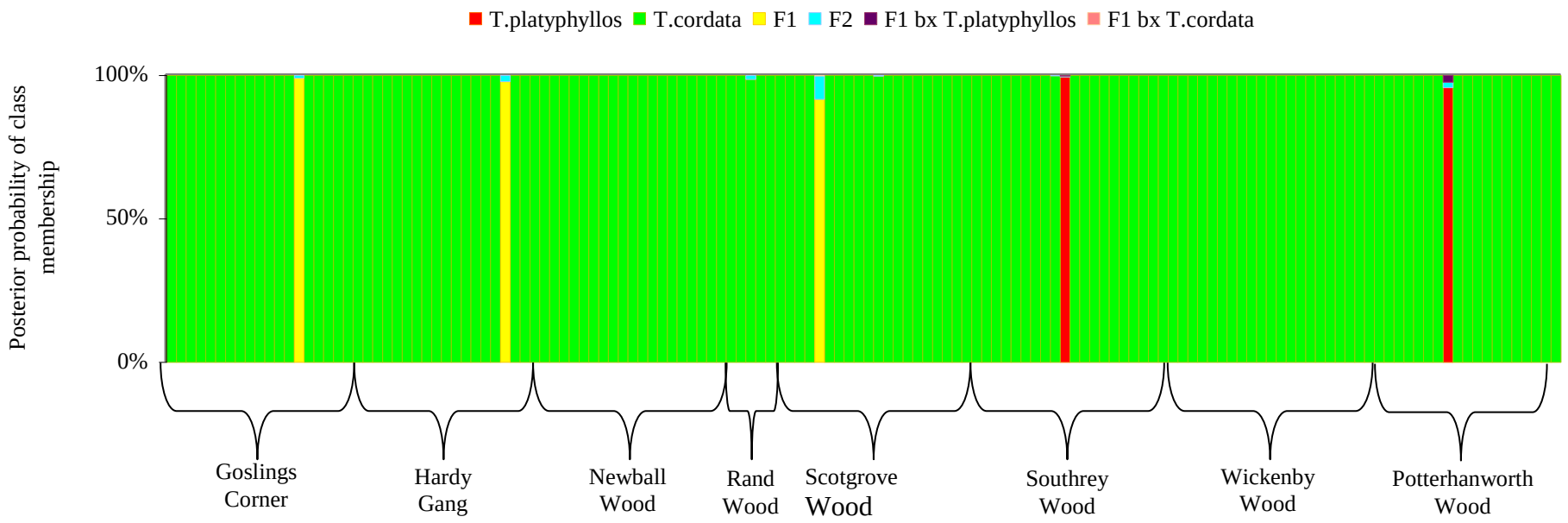
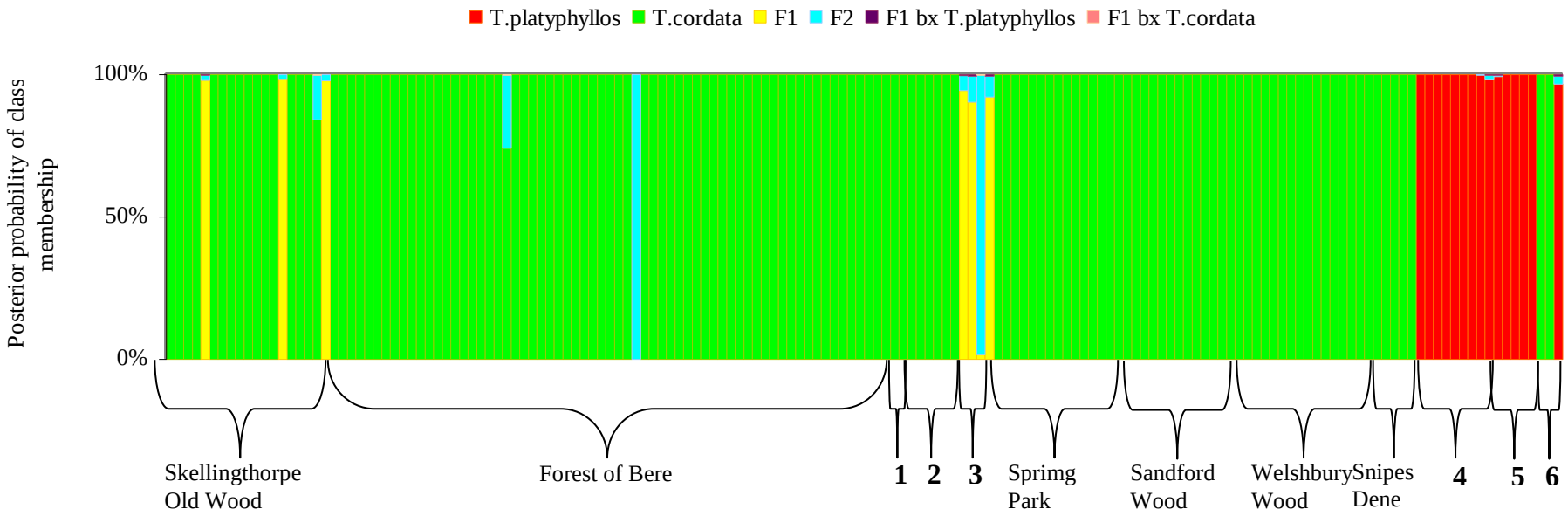


Figure 4.4 (**continued**) Assignment of complete data set of all trees to the population classes: pure *T. cordata*, pure *T. platyphyllos* or to hybrid classes F1 hybrid, F2 hybrid, F1 backcrossed to *T. cordata* or F1 backcrossed to *T. platyphyllos* by posterior probability of class membership. Each bar represents one individual tree and the trees are arranged in their woods (continued).

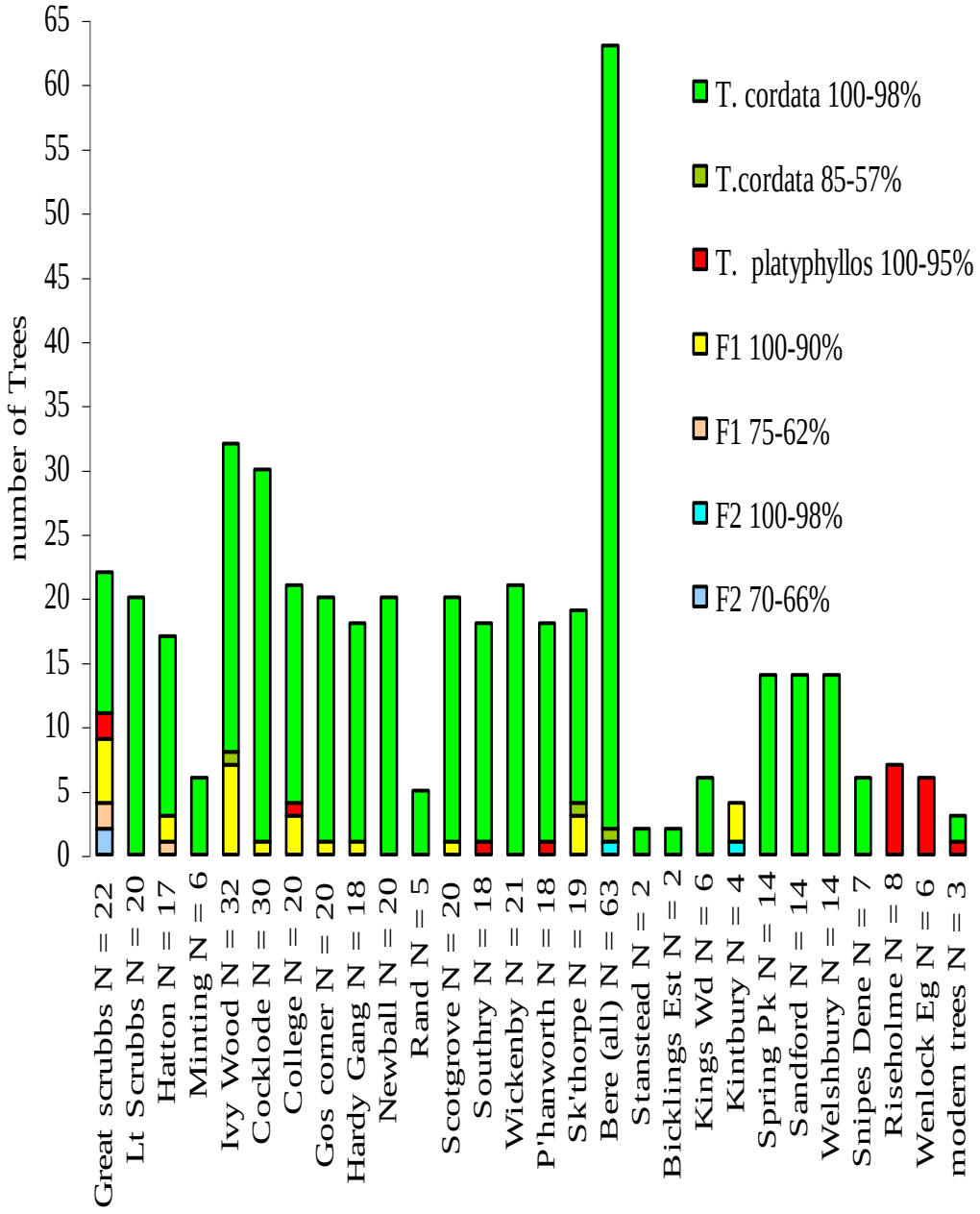


Numbered Woods

1	Bickling Estate
2	Kings Wood
3	Kintbury
4	Riseholme Park
5	Wenlock Edge
6	Various planted trees
7	

Figure 4.4 (continued) Assignment of complete data set of all trees to the population classes: pure *T. cordata*, pure *T. platyphyllos* or to hybrid classes F1 hybrid, F2 hybrid, F1 backcrossed to *T.* cordata or F1 backcrossed to *T. platyphyllos* by posterior probability of class membership. Each bar represents one individual tree and the trees are arranged in their woods.

Figure 4.5 *T. cordata, T. platyphyllos*, F1 and F2 tree assignment to population group by wood. The trees that are assigned by percentage with less confidence are also indicated. The 17 woods from the Forest of Bere are grouped as one area having a total population size of 63 trees, an average of 3.7 trees per wood.



4.3.3 Genetic Diversity

The data for genetic diversity was obtained from 20 separate woods each providing between 9 and 29 samples. To estimate the influence on the genetic diversity of the presence of *T. platyphyllos* and *cordata / platyphyllos* hybrids in the woods, the *T. cordata* population (*T. cordata* N = 338) results were compared with those from the same woods with all the sampled trees included (All *Tilia* N = 383). Estimates of genetic diversity within the populations are shown in table 4.4.

From the analysis of populations with only *T. cordata* trees considered, 70 alleles were detected, compared with 99 alleles from the all *Tilia* populations. Private alleles were detected in 11 of the *T. cordata* populations but in only eight of the all *Tilia* populations, with three private alleles being detected in the Wenlock Edge *T. platyphyllos* population. Regionally, the Lincolnshire population contains the greatest number of private alleles, with 20 (86 %) of the 23 private alleles detected among the *T. cordata* trees and 15 (88 %) of the 17 private alleles if the population of all *Tilia* are considered. A paired sample t-test, however, indicates that there is no significant difference between the numbers of private alleles in each group ($t = -1.40$, $P = 0.18$).

The mean number of alleles over all loci observed in the *T. cordata* populations was 3.7 (standard error s.e. 0.10) and ranged between 4.8 (s.e 0.66) for Ivy Wood and 2.8 (s.e. 0.42) for Spring Park. With all *Tilia* included in the data the mean number of alleles increased to 4.3 (s.e. 0.2), with a maximum number of 6.1 (s.e. 0.78) occurring in Great Scrubbs Wood, where only 45% of the trees sampled were considered to be pure *T. cordata*. A paired sample t-test indicates that the mean number of alleles / locus differs significantly between the two sets of data (*T. cordata* vs all *Tilia*; $t = 3.73$, $P = 0.0014$).

Except for Poterhanworth Wood and Lymington Purlieu, observed (Ho) and expected heterozygosity (He) both increased when all *Tilia* were included in the populations, as did the value of F, the fixation index. These overall increases were significant between the two groups (He: t = 2.94, P = 0.0085; Ho: t = 2.54, P = 0.020; F: t = 2.26, P = 0.035)). The mean value for the genetic diversity (He) over *T. cordata* populations was 0.49 (s.e. 0.007) with a range from 0.57 to 0.41 and for all *Tilia* populations mean He = 0.52 (s.e. 0.016) with a range from 0.73 to 0.41. Expected heterozygosity was greatest in Great Scrubbs Wood with values of 0.57 (s.e. 0.064) for *T. cordata* only and 0.73 (s.e. 0.029) for all *Tilia*. The minimum value for He occurred in Spring Park, 0.41 (s.e. 0.078) where only *T. cordata* was sampled. The genetic diversity of *T. cordata* in the 12 populations that were found to also contain *T. platyphyllos* and /or hybrids was significantly higher than for the eight populations where only *T. cordata* had been identified (Na: t = 2.92 P = 0.009; Ne: t = 3.01 P = 0.004; Np: t = 2.35 P = 0.030; He: t = 3.42, P = 0.0030). Heterozygote excess was observed in five of the woods but in these cases F was not significant. Heterozygote deficiency, where values for the fixation index were positive and significant (P< 0.05), occurred in eight of the *T. cordata* populations but in 12 of the all *Tilia* populations. The fixation index F was < 0.1 in 15 woods, irrespective of population composition, with a mean value of 0.04 (s.e. 0.017, range 0.18 to -0.12 for *T. cordata* and mean 0.05 (s.e. 0.018, range 0.23 to -0.12) for all *Tilia*.

With the trees grouped regionally into much larger populations, the proportion of *T. platyphyllos* and *cordata* / *platyphyllos* hybrid trees in each region was low. In Lincolnshire *T. platyphyllos* and *cordata* / *platyphyllos* hybrid trees accounted for 10.5 % of the total population, with 271 *T. cordata* trees sampled together with 32 trees that were not pure *T. cordata*. Values for Ne, Ho and He were similar for all regions and F was

significant and positive in the two larger regional groups of the south and Lincolnshire (south *T. cordata* F = 0.12, s.e. 0.018; all *Tilia* F = 0.12 s.e. 0.020; Lincolnshire *T. cordata* F = 0.12, s.e. 0.045; all *Tilia* F = 0.09 s.e. 0.047). However, F was not significant for the smaller west and central populations.

T. platyphyllos appeared more genetically diverse than *T. cordata*. When compared with *T. cordata* populations the combined group of *T. platyphyllos* from Wenlock Edge and from Riseholme Park gave higher mean levels of genetic diversity for effective alleles and private alleles as well as for observed and expected heterozygosity (*T. platyphyllos*: Ne = 2.7, Np = 0.3, Ho = 0.62, He = 0.58; *T. cordata*: Ne = 2.45, Np = 0.1, Ho = 0.47, He =0.49). However, as the combined *T. platyphyllos* population group size was only 12, the significance of these results was not determined.

Table 4.4 Genetic diversity characteristics, derived from ten microsatellite markers, of both 20 populations and four regions. *T. cordata* populations are compared with the same populations with all *Tilia* trees sampled (*T. cordata, T. platyphyllos* and hybrids). Also included is a combined group containing trees collected as *T. platyphyllos* from Wenlock Edge and Riseholme Park (planted trees). The mean value over all loci is are given for Na, Ne, Np, Ho, He and F as well as a total mean over all populations and loci.

	No. of samples analysed (N)		Na mean		Ne mean		Np		Ho mean		He mean		F mean	
Population	***T. cordata***	**All *Tilia***	***T. cordata***	**All *Tilia***	***T. cordata***	**All *Tilia***	***T. cordata***	**All *Tilia***	***T. cordata***	**All *Tilia***	***T. cordata***	**All *Tilia***	***T. cordata***	**All *Tilia***
Gt. Scrubbs	9	20	3.7	6.1	2.7	4.1	0.0	0.2	0.553	0.651	0.567	0.727	0.013	0.104***
Hatton	14	17	3.8	5.1	2.5	2.9	0.1	0.1	0.480	0.512	0.520	0.569	0.121*	0.126**
Ivy	23	29	4.8	5.5	2.6	2.9	0.5	0.0	0.469	0.520	0.511	0.567	0.049*	0.051**
Lt. Scrubbs	20	20	3.9	3.9	2.5	2.5	0.1	0.1	0.489	0.489	0.491	0.491	-0.029	-0.029
Cocklode	29	30	4.1	4.7	2.7	2.8	0.1	0.0	0.490	0.501	0.513	0.525	0.004	0.009
College	16	20	3.6	5.2	2.6	3.0	0.0	0.0	0.408	0.455	0.503	0.582	0.176***	0.227***
G. Corner	19	20	3.7	4.3	2.5	2.6	0.1	0.1	0.486	0.492	0.492	0.509	0.001	0.020
H. Gang	17	18	4.0	4.5	2.5	2.5	0.2	0.2	0.460	0.463	0.502	0.515	0.055*	0.072**
Newball	20	20	3.8	3.8	2.6	2.6	0.1	0.0	0.445	0.445	0.480	0.480	0.083*	0.083*
Scotgrove	19	20	3.9	4.7	2.4	2.6	0.1	0.0	0.405	0.423	0.484	0.511	0.155***	0.141***
Southrey	19	20	4.1	4.9	2.5	2.7	0.3	0.3	0.492	0.503	0.518	0.541	0.059	0.074*
Wickenby	21	21	3.9	3.9	2.4	2.4	0.0	0.0	0.445	0.445	0.490	0.490	0.083*	0.083*
Phanworth	17	18	3.7	4.4	2.5	2.7	0.2	0.1	0.510	0.498	0.510	0.539	-0.005	0.069*
Sk.Old Wood	16	19	3.6	4.5	2.3	2.6	0.0	0-0	0.414	0.457	0.464	0.521	0.085*	0.090**

(N sample size, Na number of different alleles, Ne number of effective alleles, Np number of private alleles, Ho observed heterozygosity, He expected heterozygosity, F fixation index (* $p<0.05$, ** $p<0.01$, *** $p<0.001$), s.e. standard error).

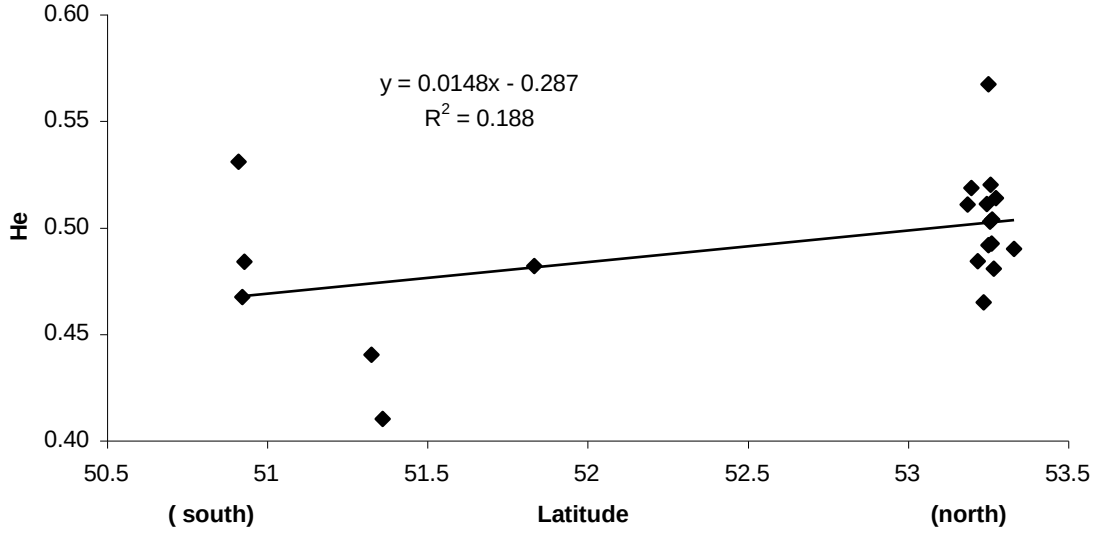
Table 4.4 continued

	No. of samples analysed (N)		Na mean		Ne mean		Np		Ho mean		He mean		F mean	
Population	***T. cordata***	**All *Tilia***	***T. cordata***	**All *Tilia***	***T. cordata***	**All *Tilia***	***T. cordata***	**All *Tilia***	***T. cordata***	**All *Tilia***	***T. cordata***	**All *Tilia***	***T. cordata***	**All *Tilia***
Lym P.	16	17	4.3	4.3	2.5	2.5	0.2	0.1	0.485	0.480	0.530	0.528	0.115*	0.118*
Gt. Lion	10	10	3.1	3.1	2.2	2.2	0.0	0.0	0.487	0.487	0.484	0.484	0.021	0.021
Hun.C.	10	10	2.9	2.9	2.1	2.1	0.0	0.0	0.523	0.523	0.467	0.467	-0.121	-0.121
Sp.Park	14	14	2.8	2.8	2.0	2.0	0.0	0.0	0.448	0.448	0.410	0.410	-0.076	-0.076
Sandford	14	14	3.2	3.2	2.3	2.3	0.0	0.0	0.425	0.425	0.440	0.440	0.044	0.044
Welshbury	14	14	3.7	3.7	2.4	2.4	0.0	0.0	0.494	0.494	0.481	0.481	-0.067	-0.067
Mean	16.85	18.55	3.73	4.28	2.45	2.62	0.10	0.06	0.470	0.485	0.493	0.519	0.038	0.052
SE	1.06	1.12	0.10	0.20	0.04	0.10	0.03	0.02	0.009	0.011	0.007	0.015	0.017	0.018
T. platyphyllos	-	12	-	4.0	-	2.7	-	0.3	-	0.622	-	0.581	-	-0.078
s.e.		0		0.49		0.34		0.15		0.082		0.054		0.107
Regions														
Lincolnshire	271	303	6.9	8.6	2.7	2.9	2.0	1.5	0.467	0.491	0.522	0.562	0.088*	0.123**
South	64	63	4.6	4.6	2.5	2.5	0.2	0.1	0.470	0.469	0.532	0.532	0.123*	0.123*
Central	20	24	3.4	4.7	2.2	2.8	0.1	0.0	0.471	0.529	0.471	0.555	-0.004	0.029
West	28	28	3.9	3.9	2.4	2.4	0.0	0.0	0.459	0.459	0.475	0.475	0.001	0.001
Mean	95.75	104.50	4.70	5.45	2.48	2.66	0.58	0.40	0.467	0.487	0.500	0.531	0.052	0.069
s.e.	59.20	66.74	0.77	1.06	0.10	0.12	0.48	0.37	0.003	0.015	0.016	0.020	0.032	0.032

N sample size, Na number of different alleles, Ne number of effective alleles, Np number of private alleles, Ho observed heterozygosity, He expected heterozygosity, F fixation index (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), s.e. standard error).

Genetic diversity of *T. cordata* populations was also investigated as a function of geographic location (figure 4.6). Although there was a small positive gradient between the *T. cordata* populations in the south of their range in Hampshire and the more northerly trees of Lincolnshire, the correlation between expected heterozygosity (He) and their geographic position, as expressed by degrees north (latitude), was not significant (R^2 = 0.187 P=0.057). Genetic diversity was also tested for decline in an east to west direction (R^2 = 0.093 P=0.19) and across the Lincolnshire Limewoods populations of trees (south to north R^2 = 0.036 P = 0.53; east to west R^2 = 0.138 P = 0.21), but none of these correlations was found to be significant.

Figure 4.6 The correlation between latitude and genetic diversity (expected heterozygosity He) of the *T. cordata* populations sampled across England. (This correlation is not significant (P = 0.057)).



4.3.4 Population structure

The results of AMOVA among the *T. cordata* populations shows that 82% of the total molecular variance occurred within individuals, with only 4% occurring among populations (table 4.5). When all *Tilia* data was included, the among population variance remained at 4%, with 80% of the variance occurring within individuals and 16% among individuals. Population differentiation showed significant values for F_{ST} ($P < 0.001$) for all population groups. Pairwise F_{ST} values for populations (appendix 2) show that values among *T. cordata* trees in Lincolnshire are all <0.054 and 31% of the pairwise values for the Lincolnshire Limewoods are not significant.

Table 4.5 Summary of the AMOVA analysis for 20 *Tilia* populations. The two population groups analysed were: -

A only *T. cordata* trees in the populations

B All *Tilia* but without *T. platyphyllos* from Riseholme and Wenlock Edge

A

T. cordata

Source of variation	**df**	**SS**	**MS**	**Est. Var.**	**%**			**P**
Among Populations	19	136.644	7.192	0.124	4%	**F_{ST}**	0.045	0.0001
Among Individuals	318	963.719	3.031	0.379	14%	**F_{IS}**	0.143	0.0001
Within Individuals	338	768	2.272	2.272	82%			
Total	675	1868.362		2.775	100%			

B

All *Tilia* (without Rh&WE*)*

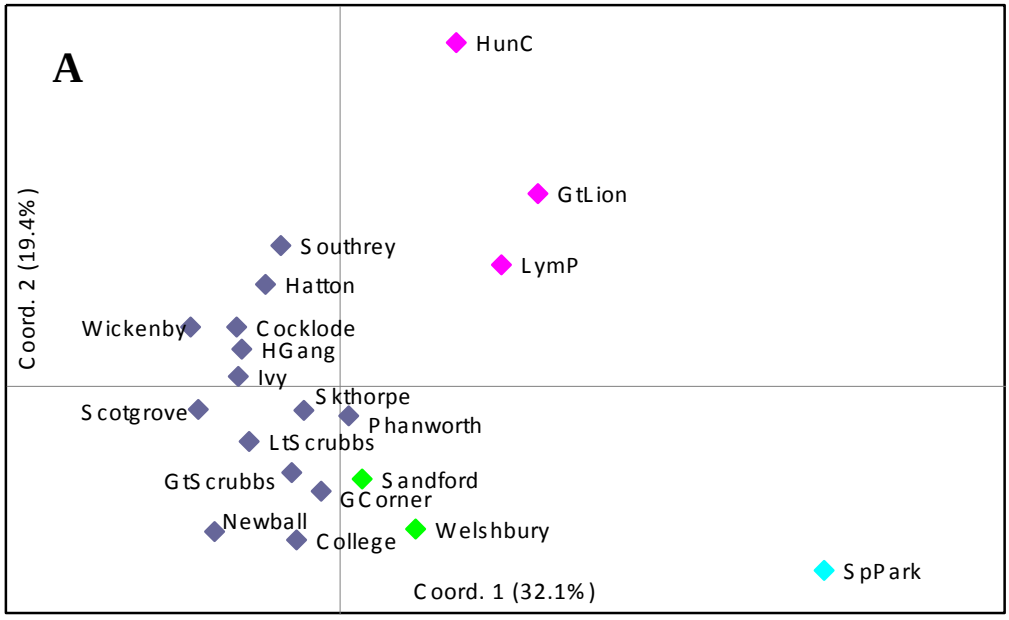
Source of variation	**df**	**SS**	**MS**	**Est. Var.**	**%**			**P**
Among Populations	3	50.034	16.678	0.114	4%	**F_{ST}**	0.040	0.0000
Among Individuals	379	1204.138	3.177	0.457	16%	**F_{IS}**	0.168	0.0000
Within Individuals	383	866.5	2.262	2.262	80%			
Total	765	2120.672		2.834	100%			

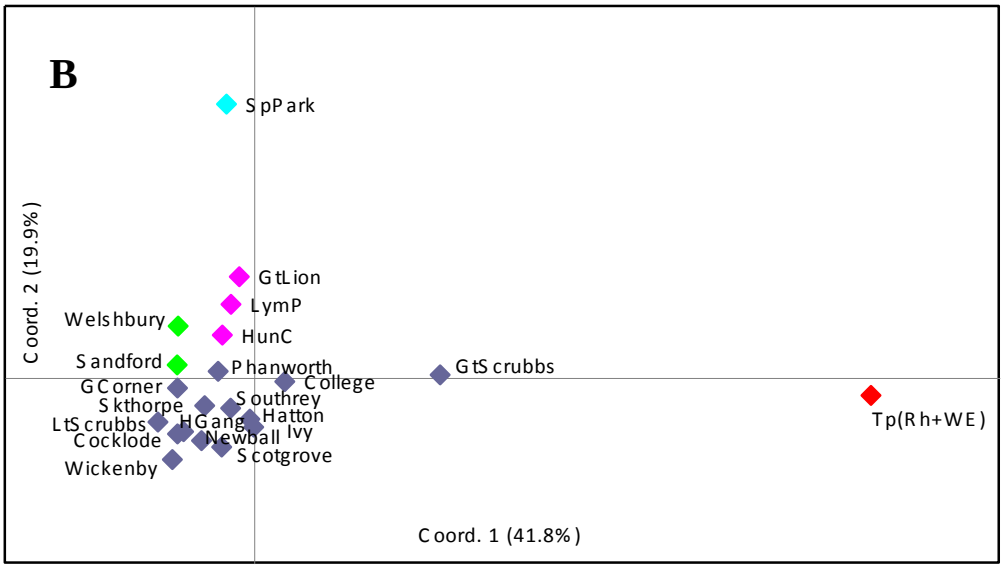
Principal coordinate analysis of population pairwise F_{ST} showed a clear separation of the *T. platyphyllos* group from the main cluster of populations that are predominantly composed of *T. cordata* (figure 4.7B). For the other populations there is regional separation, with Lincolnshire populations grouping together and being more similar to the populations from the West (Welshbury and Sandford Woods). Spring Park, a London wood, does not group with the other *T. cordata* woods even though only *T. cordata* were identified there. The trees from the Forest of Bere all group together (figure 4.7). Great Scrubbs, the wood with the highest proportion of *T. platyphyllos* and hybrid trees within its population, moves from a position within the main cluster, when only *T. cordata* tree diversity is considered (figure 4.7A), to a position outside the main cluster and more towards the *T. platyphyllos* group when all *Tilia* are included (figure 4.7B). The first two principal coordinate axes account for slightly more of the total variation when all the trees are included (*T. cordata* axis 1 = 31.0% and axis 2 = 19.4%; All *Tilia* axis 1 = 41.8% and axis 2 = 19.9%).

Figure 4.7 Principal coordinate analysis of pairwise F_{ST} between *T. cordata* populations compared with pairwise F_{ST} of the same populations including the *T. platyphyllos* and hybrids found within those woods.

A.) 20 wood populations with putative *T. cordata*. The first two axes explain 32.1% and 19.4% of the total variation.

B.) 21 wood populations with *T. cordata*, *T. platyphyllos* and hybrid trees. The first two axes explain 41.8% and 19.9% of the total variation.





◆ Lincolnshire — Including all Lincolnshire Limewoods
◆ South — Forest of Bere
◆ West — Welshbury and Sandford Woods
◆ Central — Spring Park
◆ *T. platyphyllos* — Combined group of trees from Wenlock Edge (WE) and Riseholme Park (Rh).

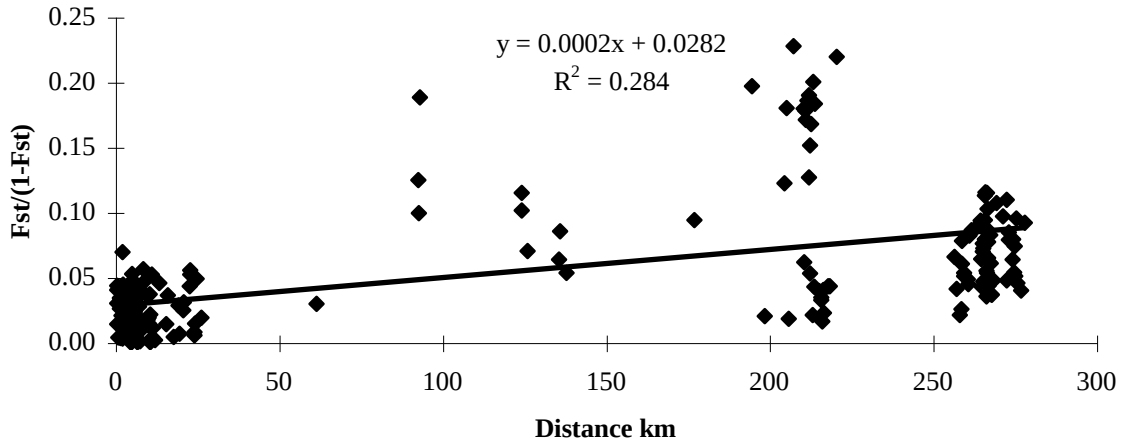
Over larger distances, Mantel tests for isolation by distance showed a weak but significant isolation by distance ($P < 0.001$) for the 20 populations (figure 4.8). Including all *Tilia* data within the populations resulted in a reduction of the R^2 value (*T. cordata* $R^2 = 0.28$, all *Tilia* $R^2 = 0.24$) but the correlation remained significant ($P < 0.001$). When isolation by distance was considered for the Lincolnshire Limewoods populations, however, the results were not found to be significant for either *T. cordata* trees alone or if all *Tilia* were included (Lincolnshire Limewoods *T. cordata* $R^2 = 0.002$ $P=0.40$, all *Tilia* $R^2 = 0.007$ $P=0.42$)) (figure 4.9)

Figure 4.8 Mantel test for the correlation between geographic distance between 20 *T. cordata* wood populations and genetic distance measured by linearised F_{ST}

A.) *T. cordata*

B.) All *Tilia* (Riseholme and Wenlock Edge *T. platyphyllos* not included)

A.)



B.)

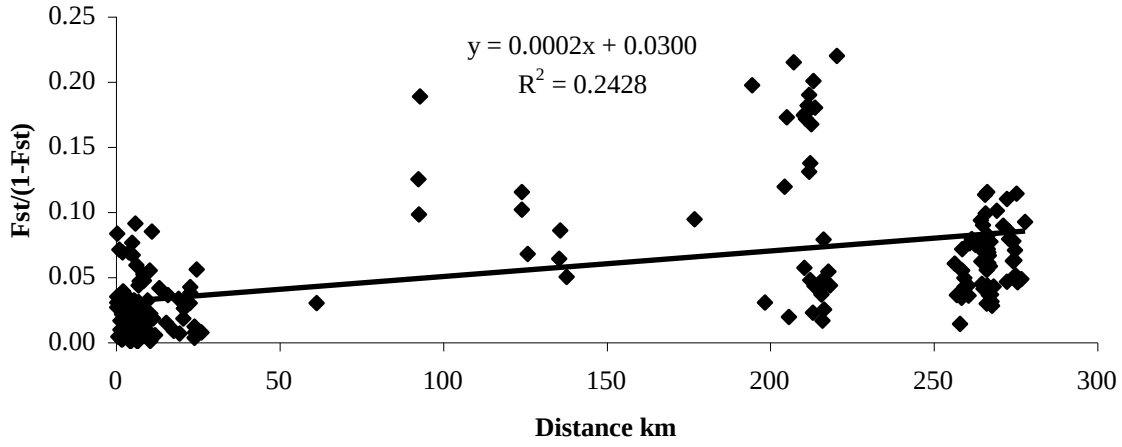
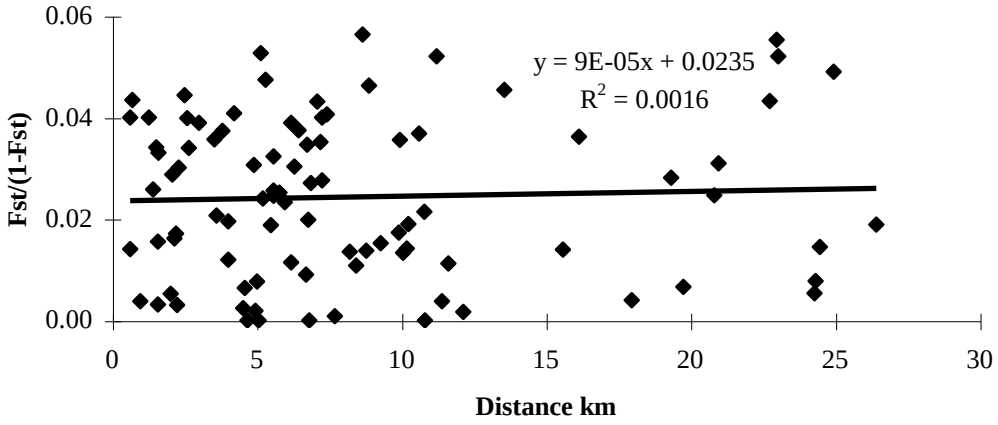


Figure 4.9 Mantel test for the correlation between geographic distance between the Lincolnshire Limewoods *T. cordata* wood populations and genetic distance measured by linearised F_{ST}

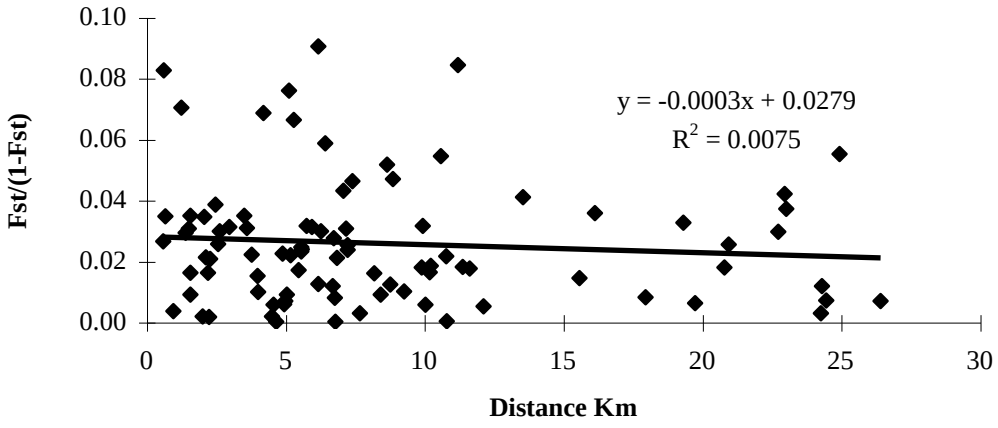
A.) *T. cordata*

B.) All *Tilia*

A.)



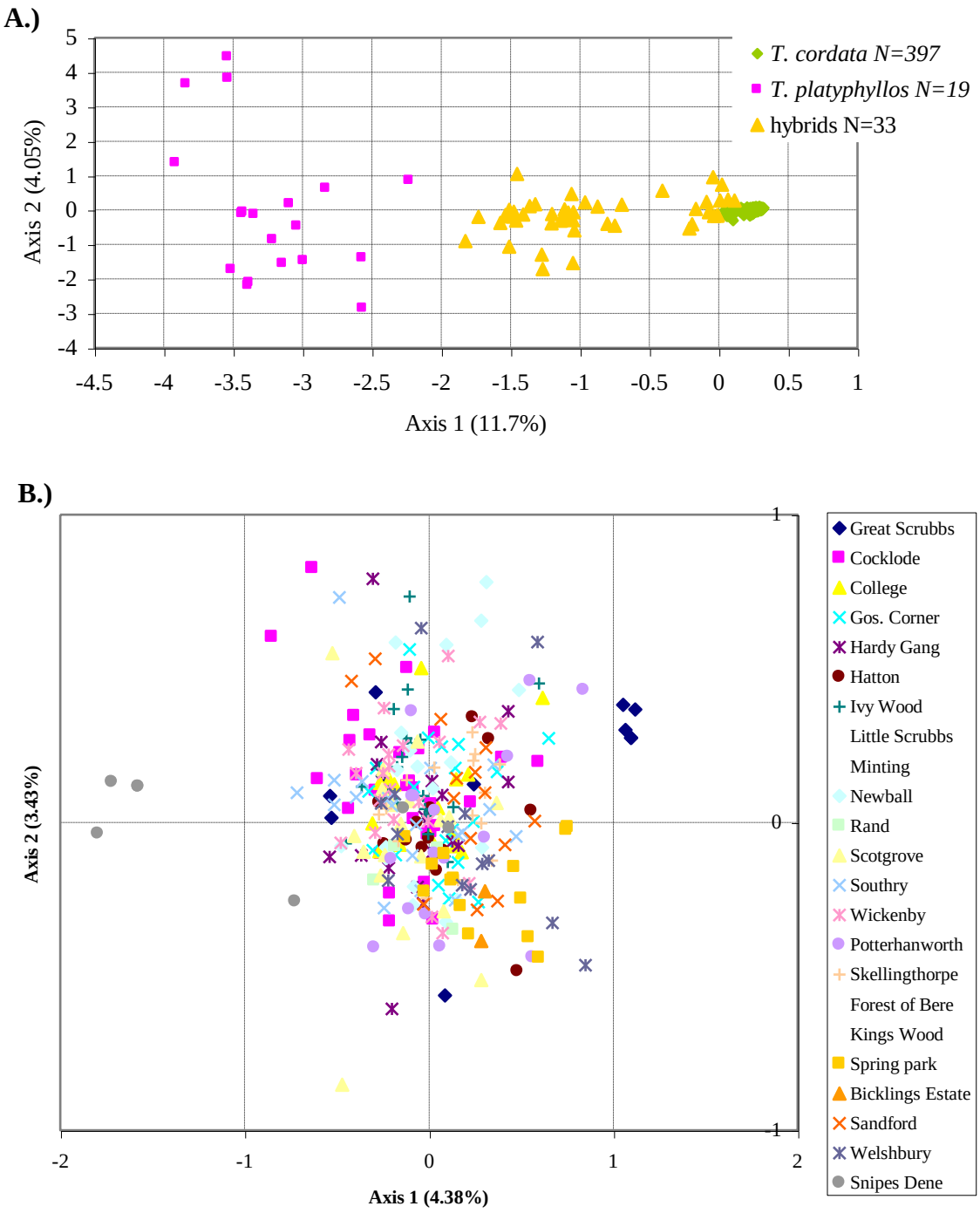
B.)



The factorial correspondence analysis (FCA) of the sampled trees (N = 449) as one group, represents 15.7 % of the total variance in the data with the first two axis of the two dimensional plot (axis 1 = 11.7 %, axis 2 = 4.05 %) (figure 4.10A). All the known *T. platyphyllos* trees from Riseholme and Wenlock Edge, as well as trees identified as *T. platyphyllos* trees by NewHybrids, have a genetic profile that separates them from *T. cordata* into a relatively widely dispersed group. In contrast, the *T. cordata* population is tightly clumped about the origin of the axis of the plot and the proximity of the points to the origin would suggest that the trees have similar genetic profiles and are relatively undifferentiated. The 33 trees that occupy the space between *T. platyphyllos* and *T. cordata* are possibly not pure *T. cordata* but are probably of hybrid origin with morphological characteristics that are intermediate between *T. cordata* and *T. platyphyllos*. Results from STRUCTURE and NewHybrids analysis support this hybrid identification (this chapter 4.3.2).

To remove the effects of the inertia of the outliers, a FCA plot of all the trees identified as *T. cordata* was also derived (figure 4.10B). Trees from 23 populations were represented within the central cluster of data points distributed about the origin. No population, however, could be uniquely separated from within this group. Plots of *T. cordata* with 3D FCA were also not able to resolve separate trees into their populations.

Figure 4.10 FCA plots indicating genetic variance between all the *Tilia* trees sampled. A.) 2D FCA plot for all *Tilia* spp. analysed. *T. cordata* (N = 397) has the least differentiated genetic profile with all trees locating near the origin of the plot. *T. platyphyllos* (N = 19) is the most diverse and hybrid trees (N = 33) locate in the space between the two groups.
B.) 2D plot of *T. cordata* (N = 397) with individual trees identified by their wood membership.

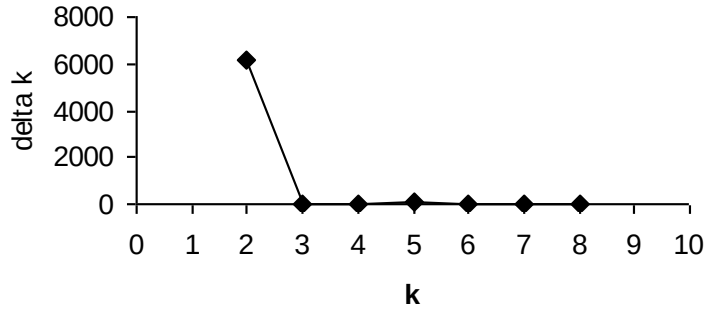


4.3.4.1 Cryptic Population Structure within the Limewoods

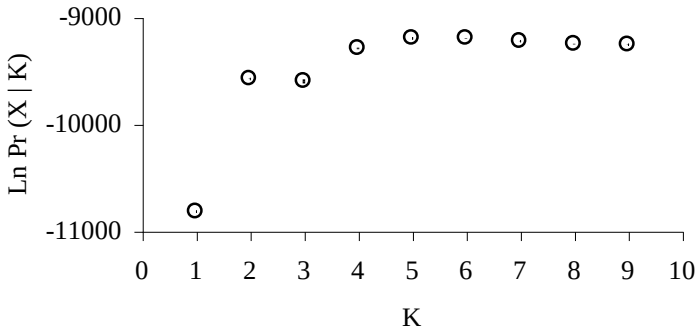
Bayesian clustering analysis of the genotype data derived from all collected tree samples was used to identify cryptic population structure among all the populations sampled. The maximum number of hierarchical population clusters (K) for the complete data set was estimated using both the method devised by Evanno *et al* (2005) and Pritchard *et al* (2000). The values of K obtained from the two approaches differed, with Evanno *et al* (2005) showing a maximum value of ΔK occurring at K = 2 and Pritchard *et al*'s suggestion, based on the maximum value of Ln Pr (X | K), giving an estimate of the number of populations as five (figure 4.11)

Figure 4.11 Comparison of estimates for the maximum number of population clusters among all the populations sampled.

A.) Evanno *et al* (2005) using ΔK



B.) Pritchard *et al* (2000) using the maximum value of Ln Pr (X | K).

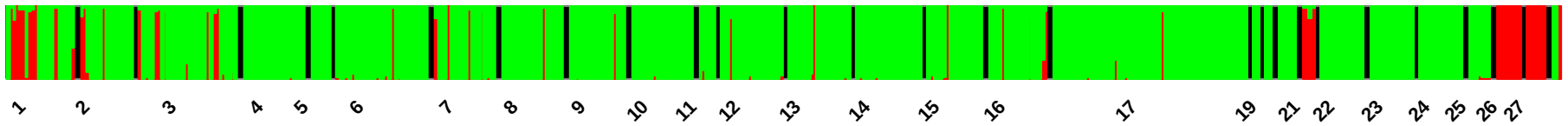


The plots of Q, the estimated membership coefficients of individuals to each population cluster, for values of K from 2 to 6, are given in figure 4.12. For each specified value of K, individual trees are arranged in their wood groups in plot a) and sorted by Q according to membership of each population cluster in plot b). For all values of K tested the *T. platyphyllos* groups of trees from the Wenlock Edge and Riseholme Park, as well as the trees from Kintbury, separate into the same cluster (in figure 4.12, for all values of K, this cluster is shown in red). Other individual trees in the data set were also found to be associated with this particular cluster and all trees associated with this cluster were identified, using NewHybrids, as either *T. platyphyllos* or a F1 or F2 hybrid (this chapter, section 4.3.2). None of the other woods, however, could be distinguished by specific cluster membership. Sorting the individual trees by Q appears to show up to five possible clusters, although, apart from the *T. platyphyllos* / hybrid cluster, none of these can be associated with any particular population. At K = 6 and above the additional level of clustering appears divided among the other clusters and cannot be interpreted as another level of population structure.

Figure 4.12 Individual tree assignment to the population structure of *Tilia* spp. derived using STRUCTURE. Plots for K=2 to 6 are shown with individual trees in a.) wood groups and b.) sorted by Q, the estimated membership coefficients of individuals to each cluster. Each cluster K is identified by colour. For values of K=7 and K=8 no addition population structure was observed. (The wood names key is included on the last page of this figure)

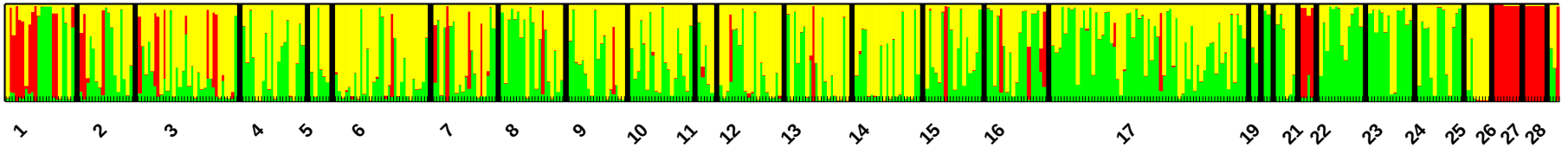
(continued)

K = 2 a) Individual trees in wood groups



K = 2 b) individual trees sorted by Q.

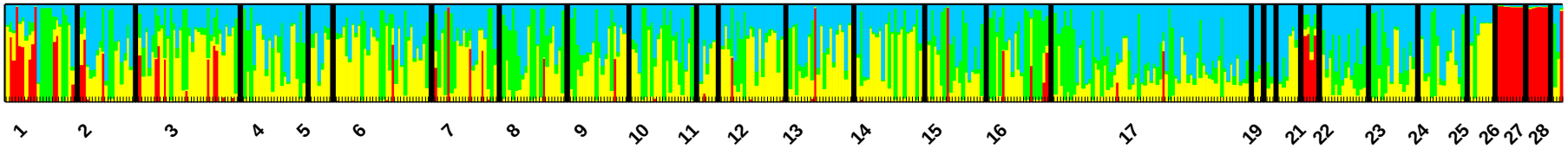
K = 3 a) Individual trees in wood groups



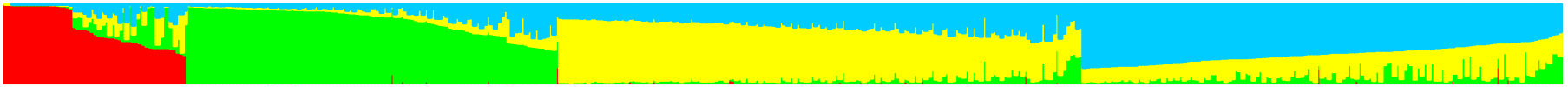
K = 3 b) individual trees sorted by Q

Figure 4.12 continued

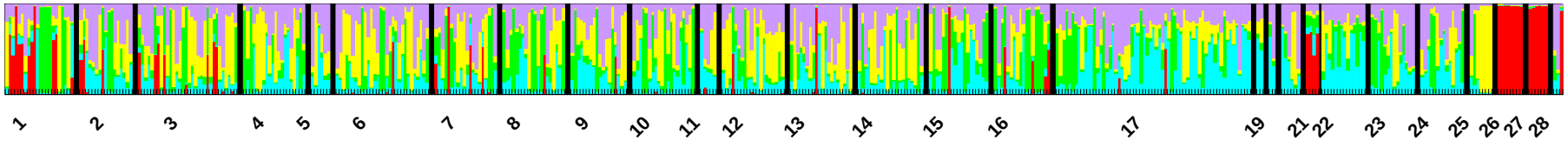
K = 4 a) Individual trees in wood groups



K = 4 b) individual trees sorted by Q.



K = 5 a) individual trees in wood groups.



K = 5 individual trees sorted by Q

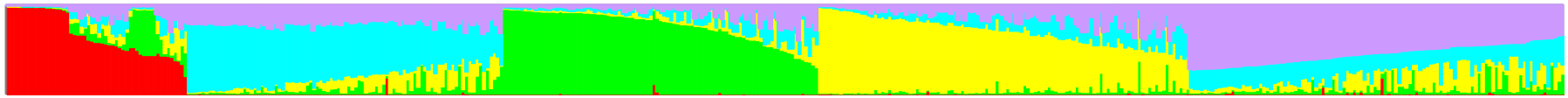
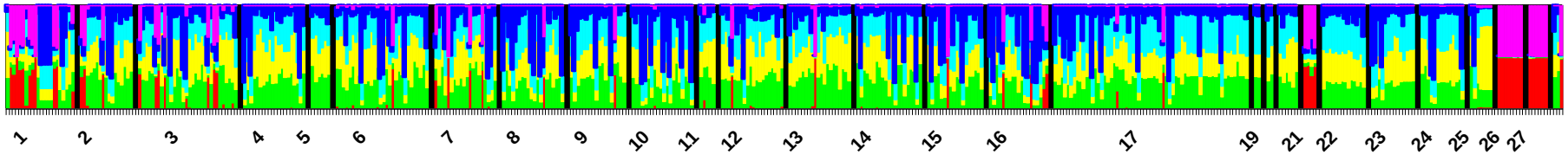
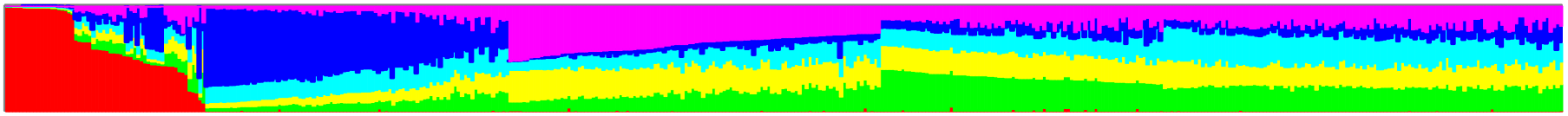


Figure 4.12 continued

K = 6 a) individual trees in wood groups.



K = 6 b) individual trees sorted by Q.



Wood group key

1	Great Scrubbs	6	Cocklode Wood	11	Rand Wood	16	Skellingthorpe Old Wood	21	Kintbury	26	Riseholme planted avenue
2	Hatton Wood	7	College Wood	12	Scotgrove wood	17	Forest of Bere	22	Spring Park	27	Wenlock Edge
3	Ivy Wood	8	GoslingsCorner	13	Southrey Wood	18	Stanstead	23	Sandford Wood	28	Recently planted trees
4	Little Scrubbs	9	Hardy Gang	14	Wickenby Wood	19	Bickling Estate	24	Welshbury Wood		
5	Minting Wood	10	Newball Wood	15	Potterhanworth Wood	20	Kings Wood	25	Snipes Dene		

Woods 1 to 14 Bardney Limewoods

Woods 1 to 16 Lincolnshire woods

Forest of Bere group contains 17 woodland areas

For K=2, STRUCTURE divided the complete data set of trees analysed into two separate clusters (figure 4.12). These two groups contained trees that were identified as either *T. cordata* or *T. platyphyllos* and all hybrids. To investigate any possible biological significance of this division, the two groups were separated and reanalysed. Group A (figure 4.13), containing 398 *T. cordata* trees with a mean membership coefficient to group A of 0.992 (standard deviation 0.024, max 0.998, min 0.739) and group B (figure 4.14), *T. platyphyllos* and hybrids, containing 54 trees with a mean membership coefficient to group B of 0.947 (standard deviation 0.090, max 0.998, min 0.425).

The maximum number of hierarchical population clusters for group A was estimated using the two methods devised by Evanno *et al* (2005) and Pritchard *et al* (2000). For the group A data set, both estimates suggest that the group could be subdivided into a maximum of three clusters (figure 4.15). The plots of Q, the estimated membership coefficients of *T. cordata* individuals (group A) to each population cluster for values of K = 2 and K = 3 do not appear to show any population structure for *T. cordata* based on specific wood membership (figure 4.13). Sorting the data by Q, the estimated membership coefficients of individuals to each cluster, indicates possibly 2 or 3 clusters, which would be in agreement with the *ad hoc* calculations of Evanno *et al* (2005) and Pritchard *et al* (2000). The distribution of these clusters, however, does not obviously correspond to any geographical location of individual woods and cluster members are distributed throughout the total population of *T. cordata* trees. At K = 4 and above the additional level of clustering appears divided among the other clusters and cannot be interpreted as another level of population structure.

Comparing the *T. cordata* mean population assignments by wood, with the Lincolnshire Limewoods and the Forest of Bere grouped as single large populations, for both K = 2 and K = 3 clusters there is little differentiation between the sampled populations from the south and the north (figure 4.16). However, if the Lincolnshire Limewoods *T. cordata* individual woods are compared with the Forest of Bere woods for K =3 (figure 4.17), although all woods contain contributions from all three clusters, differences in the proportions of population assignment to each population classes can be observed. The woods in the Lincolnshire Limewoods appear to be more similar to each other than to the woods from the Forest of Bere.

For the *T. platyphyllos* and hybrid trees included in group B the maximum number of hierarchical population clusters for the data set was estimated using both the method devised by Evanno *et al* (2005) and Pritchard *et al* (2000). Estimates using Evanno *et al* (2005) suggest that group B could be subdivided into a maximum of two clusters whereas Pritchard *et al*. suggests a maximum of four population clusters. To explore this, group B was subdivided (figure 4.14) using STRUCTURE at the k =2 level into two further clusters. Group C (20 trees, mean membership coefficient = 0.916, s.d. = 0.159, max = 0.995, min = 0.495) and group D (34 trees, mean membership coefficient = 0.981, s.d. = 0.012, max = 0.995, min = 0.95). All the trees identified by NewHybrids as being *T. platyphyllos* and all the trees identified during collection as being *T. platyphyllos* formed Group C, while Group D was comprised of all the trees identified by NewHybrids as F1 and F2 hybrids. Group C was further divided into two clusters, group E (15 trees, mean membership coefficient =0.952, s.d. = 0.0432, max = 0.981, min = 0.815) and group F (five trees, mean membership coefficient = 0.935, s.d. = 0.103, max = 0.982, min = 0.75). *T. platyphyllos* from both Wenlock Edge and Riseholme were found to be present in both groups E and F.

Figure 4.13 The breakdown of population group A containing 398 putative *T. cordata* trees using STRUCTURE. The population assignment estimates for both K = 2 and K=3 is compared. The key to the wood names is as in figure 4.14.

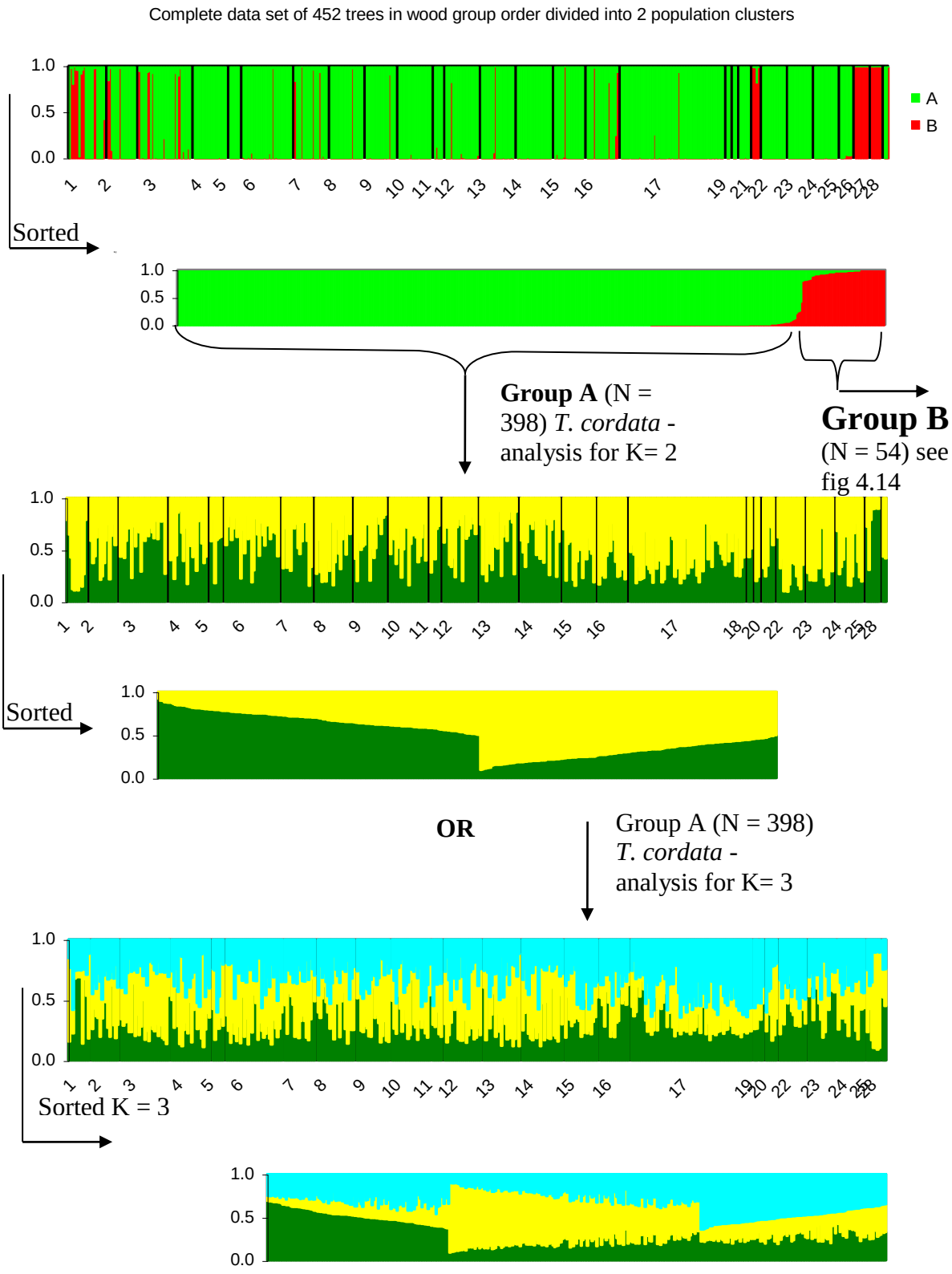


Figure 4.14 The breakdown of population group B (54 trees) using STRUCTURE at the K = 2 level. The population was broken down into 3 population clusters. Group D (34 trees) comprising of all the trees identified as F1 and F2 hybrids by NewHybrids and group C which could be further divided into group E (15 trees) and F (5 trees) containing only *T. platyphyllos*.

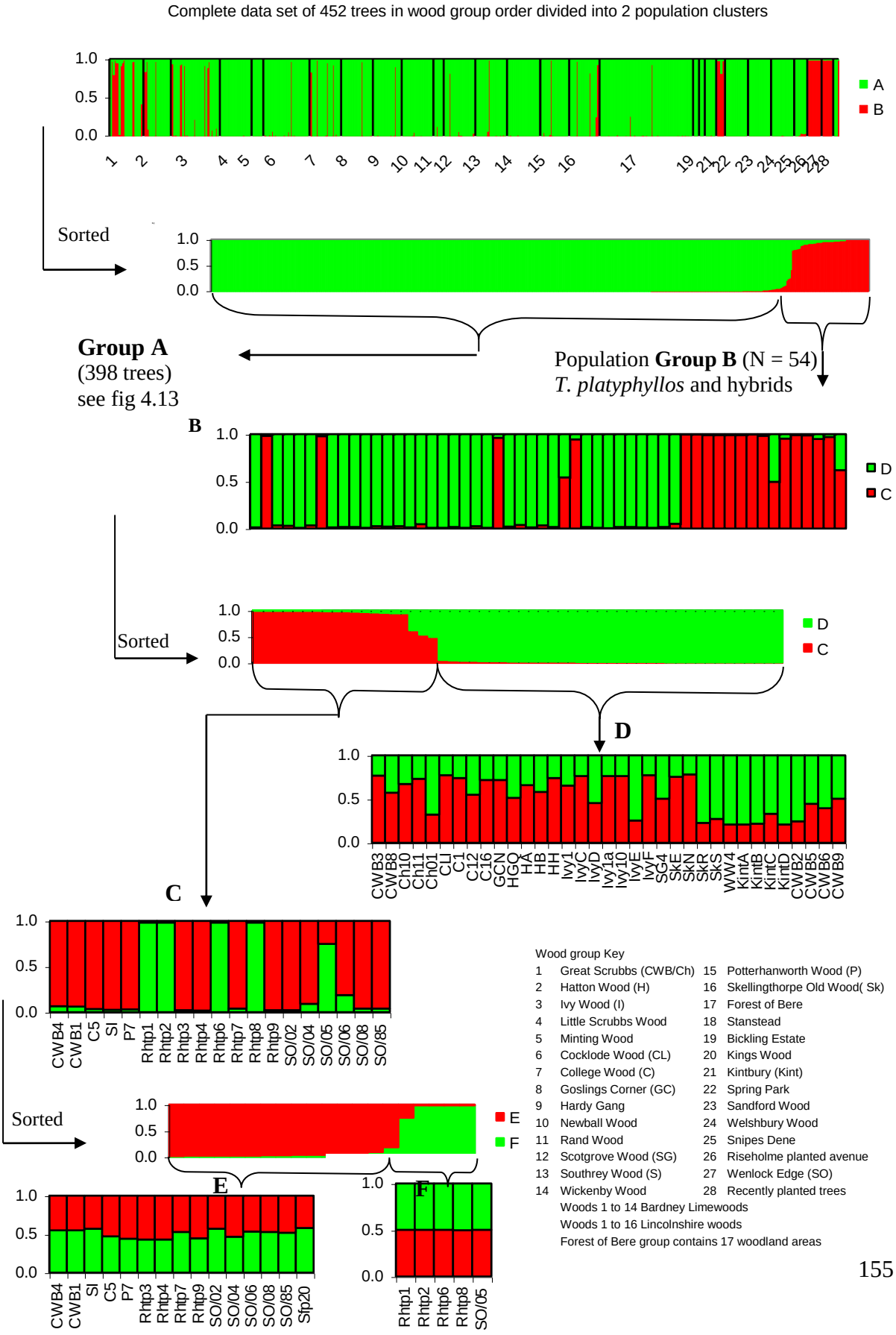
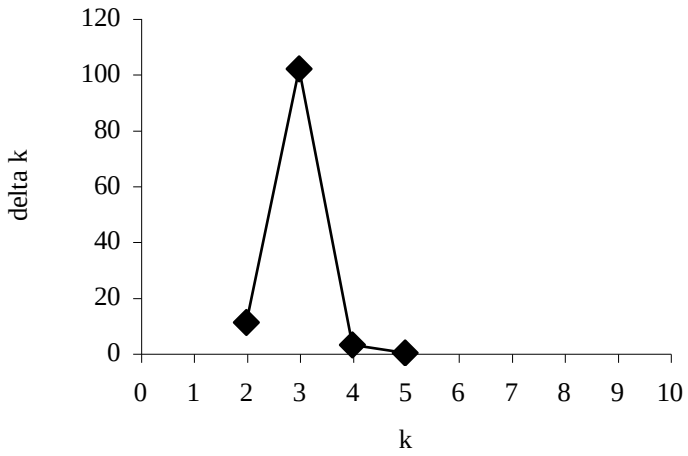


Figure 4.15 Comparison of estimates for the maximum number of population clusters for putative *T. cordata* data set (group A).

a) Evanno *et al* (2005) using ΔK

b) Pritchard *et al* (2000) using the maximum value of Ln Pr (X | K).

Both methods give the most likely maximum estimate of population clusters as three.



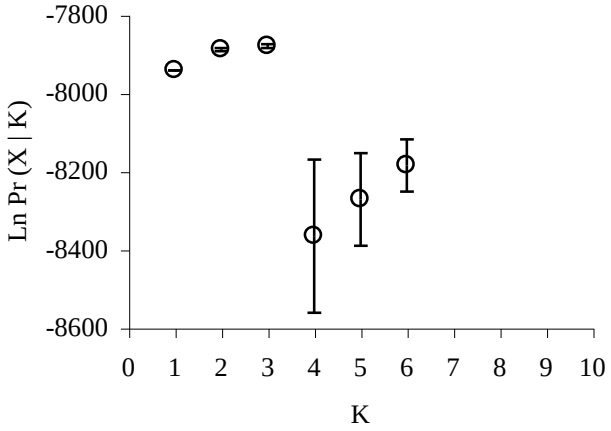


Figure 4.16 The Assignment of wood groups to population classes by average posterior probability to each population cluster. The assignment to either K =2 or K = 3 population clusters is compared for all the woods, with the two large areas of woods, the Bardney Limewoods and the Forest of Bere, shown as single populations. The size of the pie charts is related to the number of trees analysed in each area (with a minimum size for small population areas). The number shown after the wood group name is the size of the population analysed in each group

K=2

K=3

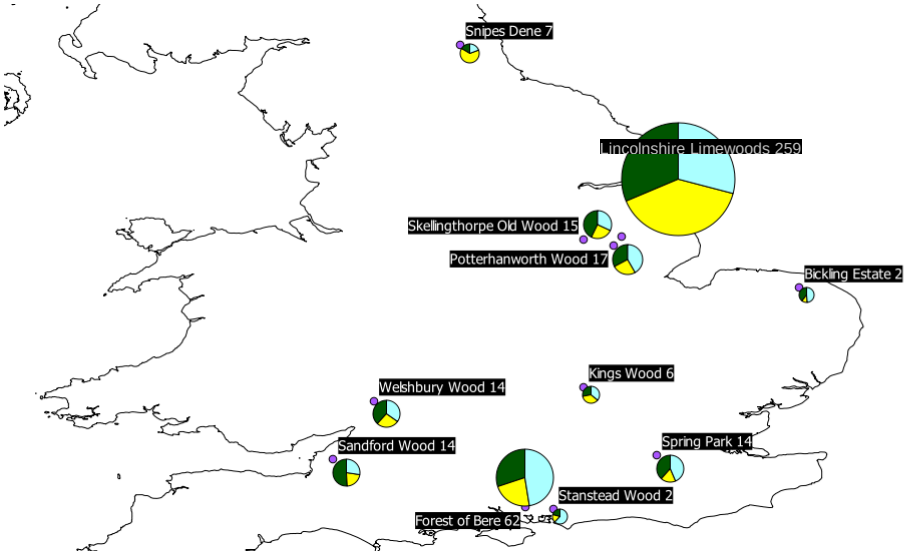
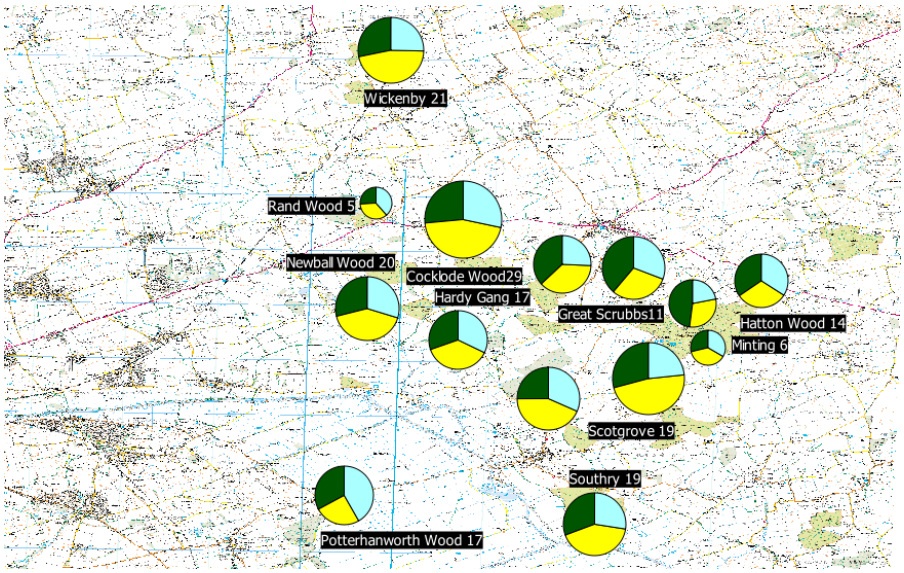
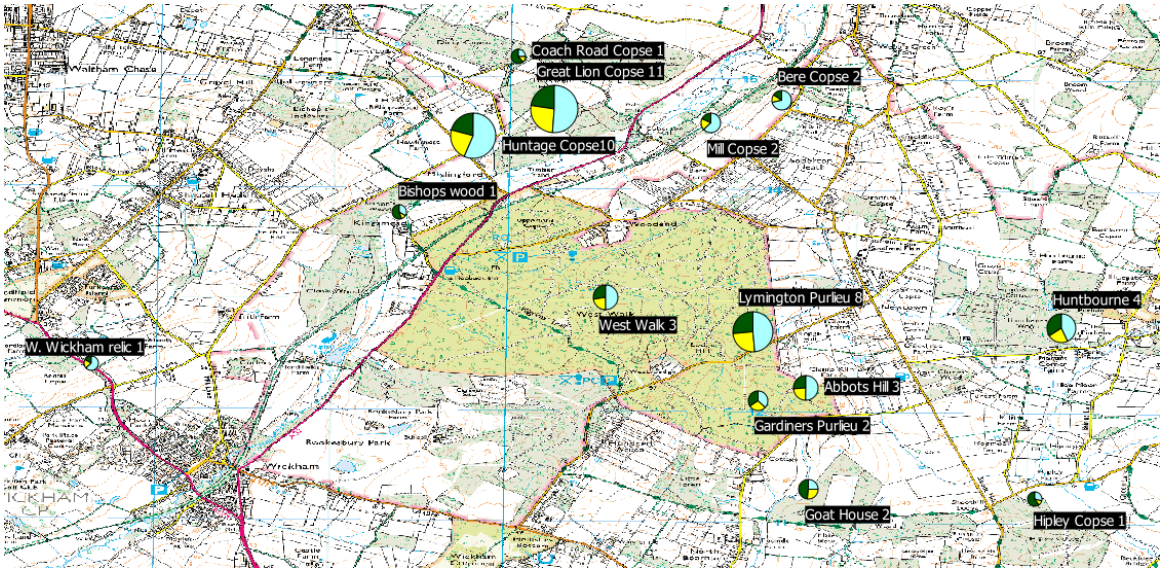


Figure 4.17 The assignment of woods of the Lincolnshire Limewoods and the Forest of Bere to population classes by average posterior probability to each for three population clusters. The size of each pie chart is related to the number of trees analysed in each area (with a minimum size for small population areas). The number shown after the wood group name is the size of the population analysed in each group.

Lincolnshire Limewoods



Forest of Bere



4.3.4.2 Fine Scale Spatial Genetic Structure

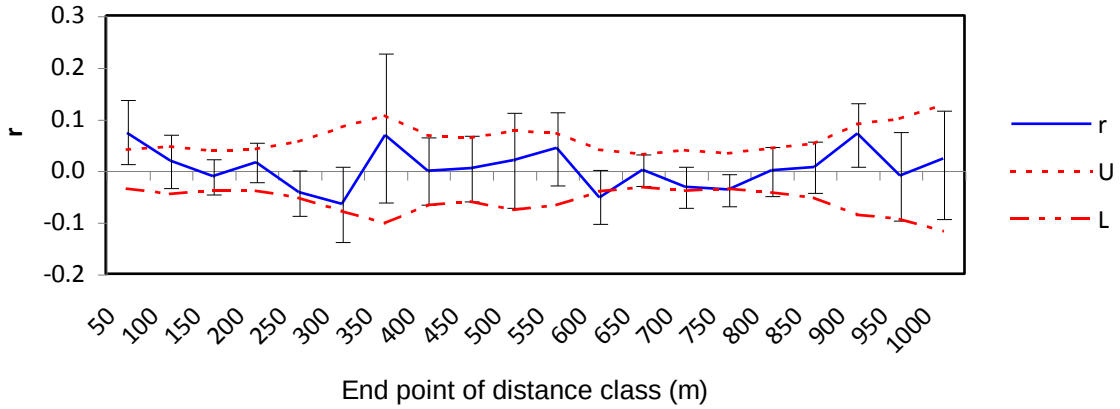
Fine scale spatial genetic structure (SGS) was determined for the trees within the Chambers Farm group of woods (Great Scrubbs, Hatton, Minting, Ivy and Little Scrubbs woods) (figure 4.18). The average distance between adjacent trees sampled in this population group was 30.2 m (range 5m to 150m). Fine scale SGS was observed but this was only significant for distances of < 100m for both population compositions. For the distance class 0 to 50 m significant SGS was weaker for the population when only *T. cordata* was analysed (auto correlation coefficient r =0.073, P<0.001) than for the analysis with all *Tilia* trees (r = 0.115, P=0.001).

Figure 4.18 Fine scale spatial genetic structure of trees from within the Chambers Farm Wood group with a distance class size of 50m. This group includes Great Scrubbs, Little Scrubbs, Hatton, Minting and Ivy woods. The autocorrelation coefficient r as a function of the distance between trees is bounded by the upper (U) and lower (L) 95% confidence interval about the null hypothesis of there being a random distribution of trees. The 95% error bars have been derived by bootstrapping over 10^4 replications: -

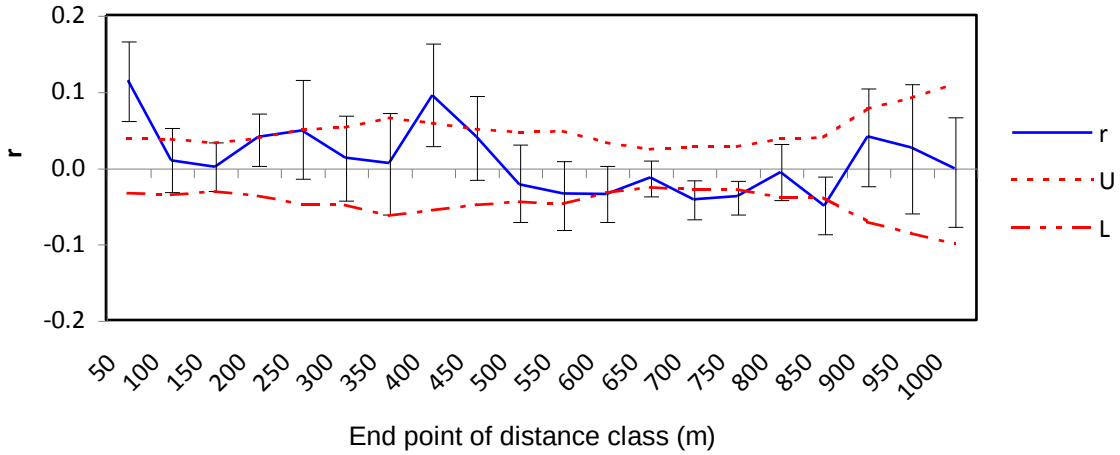
A.) *T. cordata* (N = 68)

B.) all *Tilia* spp. (N =85)

A.)



B.)



4.4 Discussion

4.4.1 Clonal Groups

T. cordata employs both sexual and asexual strategies for regeneration, with the process of self-cloning being an effective way to extend the lifespan and spread of the trees. Natural vegetative regeneration of *T. cordata* results from the production of large quantities of new shoots that arise when stems are cut, as by coppicing or by animal browsing, or when old stems collapse. Fallen trees and branches that touch the ground can also root, giving rise to clones to that can form linear rows of new trees (Pigott, 1991). The sampling distance used in this study (typically > 30m, mean sampling distance 63 m) was chosen to reduce the likelihood that sampled trees would be clones and therefore bias the estimates of genetic variability and population structure (Balloux *et al*., 2003; Pilot *et al*., 2014). In some areas, however, closer sampling was used and groups of clones were detected within the Lincolnshire Limewoods. Data from all ten *T. cordata* microsatellite loci were required, however, to provide sufficient resolution to identify clones from a sample population that might also include siblings (PIsibs< 0.0023). For a study into the clonal populations of wild strawberries (*Fragaria virginiana* Duchesne), with more variable microsatellites, five loci were found to be sufficient (Wilk *et al*., 2009).

As well as identifying trees with identical genotypes as clones, Vaughan *et al*. (2007a) also identified *Prunus avium* L. as ramets of the same genet if, as a result of somaclonal mutation occurring during vegetative propagation, they differed at one allele at one repeat. For *T. cordata*, three groups were identified in this way and were considered to be clonal.

As a result of limited sampling, it was unlikely that all trees in the genets were identified. However, in the largest clonal group, found in Great Scrubbs Wood where three ramets were identified, the mean distance between the trees was 18.4 m, suggesting that by triangulation between the three trees the genet would cover an area of at least 165 m^2. Vaughan *et al*., (2007b) identified much larger genets of wild cherry (*P. avium*.) in unmanaged ancient woodlands, the largest of which contained 65 ramets and covered an area of 850 m^2. For cherry, where clones are formed from the production of suckers, it was suggested that a sampling distance of 100m was required to avoid sampling clones. Within the Lincolnshire Limewoods there is no evidence of the genets being so extensive and a sampling distance of 50 m would be likely to avoid ramets from the same genet.

Clonal groups of trees were also identified in the trees collected from outside Lincolnshire. For trees in Spring Park and Snipes Dene these had been identified on collection as, potentially, members of the same clonal group. Results from the analysis with ten microsatellite loci were in agreement with this identification. The clonal groups of trees from Kintbury and Riseholme Park were all planted and, as a result, were more likely to have originated from cuttings or layering (Pigott, 1992). Clonal *T. cordata* in Welshbury wood was identified with a similar mean spacing (6.75 m) to that observed among the three genets identified among the Lincolnshire Limewoods populations.

All of the woods in the study have, at some time in the past, been managed by coppice rotation and the maintenance of coppice stools may have precluded the formation of large genets that could have arisen from the establishment of shoots from fallen trees and layered branches. The establishment of a minimum intervention wood within the Lincolnshire Limewoods, at Hatton Wood, may, over time, facilitate the expansion of

genets and provide some estimation of the potential for this type of regeneration. The sampling carried out for this study was of insufficient intensity to detect small genets or all trees within a genet. More intense sampling of an area of ancient woodland would give a more accurate estimate how both the extent and structure of genets and the establishment of seedlings contribute to regeneration within the wood.

Sexual reproduction of the trees gives rise to an increase in genetic diversity within the seedling population as well as the potential to disperse seeds over a wider area than generally encompassed by a ramet. With clonal populations, adaptive evolution is limited but growth can continue in situations that may be unfavourable for seedlings (Eckert, 2002; Wilk *et al*., 2009). The presence of extensive clones within woodland can result in reduced genetic diversity that may leave populations unable to adapt to ecological threats such as climate change or disease. This was particularly evident with *Ulmus procera* Salisb. where the extensive clonal population was found to be particularly susceptible to disease (Gil *et al*., 2004).

4.4.2 Identification of *T. platyphyllos* among the *T. cordata* Populations and Hybridisation between *T. cordata* and *T. platyphyllos*

Hybridisation of *T. cordata* and *T. platyphyllos* has been reported in studies of natural populations using molecular markers (Fromm and Hattemer, 2003; Liesebach and Sinko, 2008; Phuekvilai, 2014; Logan *et al*., 2015) and using leaf morphology (Pigott, 1969; Wicksell and Christensen, 1999). Molecular markers have been found to produce higher estimates of hybridisation than that achieved using morphological features (Gonzalez-Rodriguez *et al*., 2004).

Using microsatellite analysis, out of the 276 trees sampled in the Lincolnshire Limewoods 10.5 % were identified as not being pure *T. cordata*, i.e. four *T. platyphyllos* (1.4%) and 25 hybrid trees (9.1%). This was a slightly higher, but not significant ($t = 1.48$ $df = 686$ $P = 0.14$), proportion than that found in the study by Logan *et al*. (2015), where 6.1% of *Tilia* spp. sampled were identified as hybrids. Within white oak populations in Europe the incidence of hybrids can be even higher, with between 11% and 31% of trees in stands identified as hybrids (Streiff *et al*., 1999).

All except one of the trees from natural forests that were identified as hybrids were located within the Lincolnshire Limewoods, with most of them being from within the Chambers Farm group of woods. There were no backcrosses from F1 hybrids to either of the pure species and, although there is the possibility that backcrosses do not occur, limited introgression has been noted at other sites (Pigott, 1969; Logan *et al*., 2015). The non-detection of any backcrosses may also be a result of insufficient sampling of the trees or the lack of variation between the trees at the ten microsatellite loci to resolve this relationship (Boecklen and Howard, 1997; Lepais *et al*., 2009).

Although the identity of most of the trees (94.7%) was determined with posterior probability levels in excess of 98%, a few lower levels of population assignment (ie <90%) were also observed. Anderson and Thompson (2002) suggest that, in populations where loci are not strongly distinctive, individuals should only be assigned to a specific genotype frequency class with caution unless the posterior probability is very high (> 0.98). Therefore, the two F2 hybrid trees from Great Scrubbs Wood (CWB6 and Ch001), assigned with <70% probability, cannot be assigned to this class with confidence. Ch001, one of the largest trees in Great Scrubbs Wood, has the probabilities F2 = 70%

and pure *T. cordata* =30% and, although identification by leaf morphology might suggest that the tree is pure *T. cordata*, the drooping inflorescences indicate that it is a hybrid (Chapter 1.5).

Within ancient woodlands outside Lincolnshire, just one tree was identified as not being *T. cordata*. This tree, an F2 hybrid in West Walk (WW 4) in the Forest of Bere, was located adjacent to West Lodge, an 18th century house within the forest, and it could be speculated that the tree may have been planted. The Kintbury samples, from Baron Court, were collected from a 140-year-old planted avenue of putative *T.* x *europaea* trees. From these eight trees three F1 hybrid and one F2 hybrid genotypes were identified. Hansen *et al*. (2014) used *T. platyphyllos* microsatellites (Phuekvilai and Wolff, 2013) to identify the genotypes used for an historical planting of a *T.* x *europaea* avenue in Denmark. Some of these genotypes were identified as still being available commercially and indicate a probable limited genetic diversity in planted *T.* x *europaea* trees. Identification of these clones facilitated the restoration planting of a new avenue.

Only *T. cordata* trees were identified in Welshbury Wood, in Gloucestershire, but one *T. platyphyllos* tree has been identified in neighbouring Flaxley Wood, 1.7 km from the sampled site. The absence of *T. platyphyllos* or hybrids in the samples taken from Welshbury Wood does not preclude their presence but, most likely, hybrids are not present as the nearest *T.* platyphyllos tree is too far away to donate pollen and the flowering time of *T. platyphyllos* and *T. cordata* is not usually synchronised.

The incidence of hybrids (Pigott, 2012; Phuekvilai, 2014;Logan, 2015) in ancient woodlands does not appear extensive. Within the Lincolnshire Limewoods hybrids were

not sampled in all woods and within the Forest of Bere only one of the sampled trees was identified as a hybrid. It would appear likely, therefore, that hybridisation within the woods, even though the woods have been in existence for the many centuries, occurs infrequently. From a conservation point of view where hybridisation occurs between naturally occurring species, the hybrids should be considered an integral part of the ecosystem and their genetic diversity preserved (Whitham and Maschinski, 1996; Allendorf *et al*., 2001). However, hybridisation with plants that have been introduced to the woods is seen as less desirable as it introduces genetic diversity that can compromise the historic genetic diversity (Ellstrand and Schierenbeck, 2000).

4.4.3 Genetic Diversity

To estimate the impact on the genetic diversity of the presence of *T. platyphyllos* and *T. cordata* x *T. platyphyllos* hybrids, the results of the analysis of all the trees collected in each wood was compared with results from the same woods with only the *T. cordata* tree data included. Private alleles were mostly detected in woods where *T. platyphyllos* and hybrids trees were also identified. Woods outside Lincolnshire, which had exclusively *T. cordata* populations, did not have any private alleles. The association of the private alleles with the presence of *T. platyphyllos* might suggest possible introgression, as three of the private alleles attributed to *T. cordata* were identified in *T. platyphyllos* or hybrid trees, but analysis of larger *T. platyphyllos* populations would be required to resolve this (Boecklen and Howard, 1997; Lepais *et al*., 2009).

The populations of trees within the Lincolnshire Limewoods where *T. platyphyllos* and hybrids were included all showed significant increases for measures of genetic diversity when compared with the genetic diversity of the populations containing only *T. cordata*.

Zalapa *et al*. (2010) found that the occurrence of *Ulmus pumila* L. and *Ulmus rubra* Muhl. hybrids within *U. pumila* populations was also associated with increases in genetic diversity.

In this study, Great Scrubbs Wood from the Lincolnshire Limewoods contained the highest proportion of F1 hybrids (7 out of 20 trees) and consequently had the maximum values for genetic diversity (He = 0.73 and Ho = 0.65). This might be expected, as F1 hybrids are heterozygous at all loci specific to that species. However, this still applies if the *T. cordata* trees from the Lincolnshire Limewoods are considered on their own, with levels of genetic diversity, such as the number of alleles, number of private alleles and expected heterozygosity, being significantly higher than in woods where hybrids and *T. platyphyllos* were never detected.. Phuekvilai (2014) determined a range of values for genetic diversity for *T. cordata* and *T. platyphyllos* from populations throughout Europe (*T. cordata* He = 0.606 to He = 0.428; *T. platyphyllos* He = 0.79 to He = 0.636) with the highest values coming from populations in the south and declining He towards the more northern populations. The range of values obtained for *T. cordata* from this study show values similar to the more northern populations. The values for *T. platyphyllos* are lower but are derived from a small population (*T. cordata* He 0.570 to 0.420; *T. platyphyllos* He = 0.581)

The fixation index, F, can hold values between 1.0 and -1.0 and is close to zero for randomly mating populations. Substantial positive values of F (i.e. heterozygote deficiency) indicate inbreeding or possibly the presence of null alleles, and negative values can be the result of negative assortative mating or a possible selection for heterozygotes. Biparental inbreeding, as a result of restricted seed or pollen dispersal, can

also give rise to heterozygote deficiency, as can the Wahlund effect caused by population subdivision (Sutherland *et al*, 2010). Positive values of F_{IS} may indicate that these are inbreeding populations but are more likely to indicate null alleles. Micro-Checker detected possible null alleles at loci tc1-45, tc2-86, and tc2-07 for *T. cordata* trees and at tc1-30 for *T. platyphyllos*. Cottrell (2003) suggested that, for mixed *Q. petraea*, *Q. robur* and hybrid woods, positive values of F may not be an indicator of inbreeding but be part of the original post glacial colonising dynamic process. The long life span of oak trees, together with prolonged woodland management by coppicing, reduces the number of generations since the recolonisation of Britain by oak after the last glacial period and, consequently, genetic equilibrium has not yet been achieved. This may also be applicable to *T. cordata*, as it has a similar history to oak and has been extensively managed by coppicing over many centuries. However, in this case, the presence of null alleles at some loci, as identified in Chapter 3 seems more probable.

Genetic diversity across all the populations sampled was not correlated with the geographical north-south gradient between the *Tilia* populations. Across Europe, widely distributed populations of plant species such as heather (*Calluna vulgaris* L.) (Rendell and Ennos, 2002), Norway spruce (*Picea abies* L.) (Tollefsrud *et al*., 2008), oak (Dumolin-Lapegue and Demesure, 1997) and *T. cordata* (Phuekvilai, 2014) have shown genetic diversity decreasing for more northern populations, possibly as a consequence of rapid expansion from glacial refugia (Hewitt, 1999). For French oak populations, however, the genetic diversity from chloroplast data was found to be only weakly correlated with latitude for *Q. robur* and not correlated for *Q. petraea* (Petit *et al*., 2002). Nor was any correlation found in white pine in two American states where genetic diversity was measured using allozymes (Rajora *et al*., 1998). Although Phuekvilai

(2014) found genetic diversity to be weakly correlated with latitude for *T. cordata* populations throughout Europe, no relationship was found for the less geographically extensive *T. platyphyllos.* In this study, the absence of a correlation for *T. cordata* is likely to be a consequence of the narrow latitude range covered by the populations sampled (51º N to 53ºN) compared with that derived from the more extensive European populations (46º N to 62ºN) examined in Phuekvilai's study (2014).

4.4.4 Population Genetic Structure

Although the *T. cordata* populations studied here are highly fragmented, high levels of within population variation were determined with AMOVA. Genetic variation is generally uniform over all the woods investigated, with only 4% ($P<0.001$) of the variance occurring between the populations. Plants that have high levels of within population variance generally have the characteristics of woody plants i.e. long lived, outcrossing and with high fecundity. These plants often have large ranges and large stable populations that are resistant to genetic changes (Hamrick *et al*., 1981; Nybom and Bartish, 2000). Even though the woods are highly fragmented, gene flow is sufficient in this long-lived insect pollinated tree to maintain high levels of within population diversity.

AMOVA and principal coordinate analysis (PCA) were used to study the spatial population genetic structure of the *T. cordata* populations. To inform conservation strategy, Sloop *et al*. (2012) used PCA to identify gene flow barriers and temporal genetic structure between populations of *Limnanthes vinculans* Ornduff in wet meadow habitats and Stacy *et al*. (2014) identified varieties of *Metrosideros polymorpha* Gaudich. using population clusters membership.

With high genetic diversity between the species, PCA of pairwise F_{ST} separates the *T. platyphyllos* populations from the predominantly *T. cordata* populations. With 31% of the pairwise F_{ST} values between Lincolnshire Limewoods populations not being significantly different from zero, these populations cluster near the origin with little differentiation. When populations with only *T. cordata* trees are considered, the populations from the Forest of Bere and Spring Park are significantly differentiated from the Lincolnshire populations. While overall genetic variance between the populations is low ($F_{ST} = 0.045$), the distinction between the population clusters groups suggests that these groups should be conserved separately.

Populations that spread from glacial refugia have been shown to experience isolation by distance (IBD) and increase in homozygosity, possibly as a consequence of leading edge colonisation during the postglacial expansion (Hewitt, 1999). Geographical features that can disrupt gene flow and habitat fragmentation can strengthen the effect of IBD (Zhao *et al*., 2013). For distances up to 30 km IBD was not evident for *T. cordata* populations. Weak but significant IBD ($P< 0.001$), however, was evident when populations over a wider area were considered (0 to 300 km) and this was also the case for Irish populations of *Fraxinus excelsior* (Beatty *et al*., 2015). With isolation by distance of *T. cordata* populations only being evident at greater distances it is most likely a consequence of the postglacial expansion into Britain at the end of the last glacial period.

Factorial correspondence analysis (FCA) is a multivariate approach to genetic differentiation in populations. Boulli *et al*. (2001) used FCA to differentiate between populations of *Pinus halepensis* Mill. using morphological features. Miranda *et al.* (2010)

found that, for 141 Spanish pear (*Pyrus communis* L.) accessions, both STRUCTURE and FCA produced three population clusters that could be associated with the geographical origin of the trees. For *Tilia* spp., although there does not seem to be evidence of any structure based on the geographical positions of populations, FCA clusters do differentiate between *T. cordata* and *T. platyphyllos*, with trees in between the two clusters having been identified by NewHybrids as hybrids. Similarly, Neophytou *et al*. (2011) used FCA to differentiate between two species of oak. In this case hybrids were found to occur in the factorial space between the *Quercus alnifolia* Poech and *Quercus coccifera* L. clusters.

With the trees identified as *T. cordata* being represented on the FCA plot in the central cluster about the origin, there appears to be little differentiation between any of the *T. cordata* trees. This is in contrast to the more diffuse and more genetically variable *T. platyphyllos* cluster containing only 19 trees derived from seven populations.

4.4.5 Identification of Cryptic Population Structure

Cluster analysis of the genotype data from all the trees sampled show, for K=2 to K=6, that the analysis separates *T. platyphyllos* and hybrids from *T. cordata*. For each additional level of clustering introduced, this partition remains. Taking the results in conjunction with the results from NewHybrid, the partitioning of the population places all the hybrid trees within the *T. platyphyllos* cluster. Within the *T. platyphyllos* cluster for K = 2, NewHybrid results suggest that trees with Q values of < 0.995 should be classed as F1 hybrids and Q < 0.400 classed as *T. cordata*. Lepais *et al*. (2009) used STRUCTURE to identify the occurrence of hybrids in oak populations and used a value of Q < 0.9 to conservatively estimate the incidence of hybrids. Logan *et al*. (2015) also used this

approach to identify *T. cordata* and *T. platyphyllos* hybrids and used a value of Q < 0.8 to differentiate the hybrids. The choice of the value of Q used to identify hybrids is a balance between misidentifying and therefore underestimating either the pure species or the hybrid. To identify first generation hybrids accurately with this method, Vaha and Primmer (2006) have estimated that between 12 and 24 loci are required, with more than 48 loci needed to identify second-generation hybrids. Therefore, in order to accurately employ this approach to identify additional levels of hybridisation in this study, it is likely that further loci would need to be developed.

For values of K from K = 2 to K = 5, the increasing number of clusters added to the complexity of the population structure but failed to match any of the clusters with geographically identified populations. More information about the population structure was derived by reanalysing the two clusters from the K =2 analysis independently. The *T. platyphyllos* and hybrids cluster could be sub-divided (K = 2) to identify the hybrids but a further division of *T. platyphyllos* did not divide the trees by population, resulting in a cluster with trees from the Wenlock Edge, the Lincolnshire Limewoods and Riseholme Park planted trees. For the *T. cordata* cluster, however, it seems most likely the correct value of K for the *T. cordata* populations is K = 1; this would be consistent with the low value obtained from the AMOVA where only 4% ($P<0.001$) of the genetic variation could be attributed to differences between the populations. The Evanno method used to estimate the most likely number of population clusters is based on a second order rate of change, and is unable to empirically identify a K = 1 population structure. Reanalysis of the *T. cordata* cluster, with K = 3, puts admixtures of all three clusters into all of the woodland populations. Small differences in the admixture distribution for the woods within the Lincolnshire Limewoods and the Forest of Bere, two geographically widely separated

groups of trees, suggests that these areas may have differing genetic profiles that may reflect an emerging isolation by distance population structure.

4.4.6 Fine Scale Spatial Genetic Structure

The detection of a statically significant fine scale spatial genetic structure (SGS) over short distances is considered, for plants, to be an indication of isolation by distance as a result of restricted seed dispersal or pollen distribution (Streiff *et al*., 1998). *Tilia* spp. are generally insect pollinated and seed dispersal is by gravity but assisted on windy days by the bract attached to the seed. Although insect pollination is potentially not as extensive as wind pollination, insects can forage widely and bumble bees have been found to travel up to 600 m (Osborne *et al*., 1999). Seed dispersal for isolated *T. platyphyllos* trees has been found to be up to 200 m on windy days (Pigott, 2012) and, adjacent to Little Scrubbs Wood *T. cordata* seedlings have been found up to 60 m away from the wood edge. Within the wood, however, seed dispersal is likely to be less extensive as the trees are more sheltered. For *Tilia*, as seed dispersal is more limited than pollen dispersal, it is more likely to affect the distribution of SGS. Only one population was investigated for evidence of fine scale SGS and even in this case, where the populations of five closely associated woods were considered, the population size (for *T. cordata* N = 68, all *Tilia* N = 85) may not be sufficient for accurate estimation of the limit of significant SGS. The method is sensitive to the sampling distance between the trees and to the distance class chosen for the analysis and Cavers *et al.* (2005) suggests that for five microsatellite loci 100 samples are required, together with a minimum of 30 pairs per distance class.

Significant SGS was observed in the Chambers Farm Wood group up to a distance of 100 m for both data sets and is consistent with seedling being identified 60 m from parent

trees in Little Scrubbs Wood. The effect of including hybrids in the *T. cordata* data did not alter the spatial genetic distance, although Valbuena-Carabana *et al*. (2007) investigated the effect of hybrids in populations of *Q. petraea* and *Q. pyrenaica* Willd. and found SGS reduced in the presence of hybrids as the kinship between neighbouring plants is reduced when a hybrid plants grows near a pure individuals. Cottrell *et al*. (2003) showed that SGS is dependent on species and woodland management for oak trees. *Q. robur* showed structuring up to 160m in a planted, managed wood but only at 80m in a natural wood that had been coppiced and *Q. petraea* showed structure up to 40m in both woods (Cottrell *et al*, 2003). Fine scale SGS can also be affected by the life stage of the plant. Zhou and Chen (2010) found that for *Ficus cyrtophylla* (Wall. Ex Miq.) Miq. significant structure occurred for the distribution of seeds and seedlings but not for saplings and adult trees. With SGS determined for only one population in the Lincolnshire Limewoods, it was not possible to detect the effect of life stage or woodland management practice on local population structure. Additional, more intense sampling of this and other populations would enable comparisons to be made, as well as increasing the accuracy and the resolution of the method.

4.4.7 Conclusion

Habitat fragmentation can theoretically result in the genetic isolation of populations as a result of reduced gene flow (Young *et al*., 1996; Sork and Smouse, 2006). For *T. cordata*, however, this does not appear to be the case. The genetic diversity of *T. cordata* is similar and high for all the populations studied and, with 82% of the genetic variance occurring within populations and only 4% between populations, there is little differentiation between the populations. Structure analysis suggests weak isolation by distance over large

distances and that the *T. cordata* populations within the Lincolnshire Limewoods appear only subtly different from the populations located within the Forest of Bere.

The ten *T. cordata* microsatellite loci developed for this study successfully identified *T. platyphyllos* and hybrid trees within the Lincolnshire Limewoods. Where populations included *T. platyphyllos* or hybrid trees, higher levels of genetic diversity were recorded for the *T. cordata* within that population, possibly as a result of introgression. Where naturally occurring hybrids occur in the woods they should be conserved alongside the *T. cordata* as they are important contributors to the total biodiversity.

If all populations are genetically similar then replanting with seed from any wood might be perceived as being suitable for conservation and restoration planting schemes. However, neutral molecular markers such as microsatellites only inform about gene flow and migration and do not show how organisms are adapted to their environment. For the Lincolnshire Limewoods, the most suitable policy for enabling expansion and regeneration within the woods would be to facilitate natural regeneration into adjacent areas and cleared conifer plantation sites. This is most likely to preserve the current biodiversity and genetic structure of the woods.

4.5 References

Allendorf, F.W., Leary, R.F., Spruell, P. and Wenburg, J.K. (2001) The problems with hybrids: Setting conservation guidelines. *Trends in Ecology & Evolution,* 16(11) 613-622.

Anderson, E.C. and Thompson, E.A. (2002) A model-based method for identifying species hybrids using multilocus genetic data. *Genetics,* 160(3) 1217-1229.

Arnaud-Haond, S. and Belkhir, K. (2007) GENCLONE: A computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Molecular Ecology Notes,* 7(1) 15-17.

Balloux, F., Lehmann, L. and de Meeûs, T. (2003) The population genetics of clonal and partially clonal diploids. *Genetics,* 164(4) 1635-1644.

Beatty, G.E., Brown, J.A., Cassidy, E.M., Finlay, C.M.V., McKendrick, L., Montgomery, W.I., Reid, N., Tosh, D.G. and Provan, J. (2015) Lack of genetic structure and evidence for long-distance dispersal in ash (*Fraxinus excelsio*r) populations under threat from an emergent fungal pathogen: Implications for restorative planting. *Tree Genetics and Genomes,* 11(3) 1-13.

Beatty, G.E., Reid, N. and Provan, J. (2014) Retrospective genetic monitoring of the threatened yellow marsh saxifrage (*Saxifraga hirculus*) reveals genetic erosion but provides valuable insights for conservation strategies. *Diversity and Distributions,* 20(5) 529-537.

Belkhir, K., Borsa, P., Chikh, L., Raufaste, N. and Bonhomme, F. (1996-2004) *GENETIX 4.05, logiciel sous Windows$_{TM}$ pour la génétique des populations.* 4.05. Université de Montpellier II, Montpellier (France): Laboratoire Génome, Populations, Interactions, CNRS UMR 5000,.

Birks, H.J.B. (1989) Holocene isochrone maps and patterns of tree-spreading in the British Isles. *Journal of Biogeography,* 16(6) 503-540.

Boecklen, W.J. and Howard, D.J. (1997) *Genetic Analysis of hybrid zones: Numbers of markers and power of resolution.* Ecological Society of America. 2611.

Boulli, A., Baaziz, M. and M'Hirit, O. (2001) Polymorphism of natural populations of *Pinus halepensis* Mill. in Morocco as revealed by morphological characters. *Euphytica*, 119(3) 309.

Cavers, S., Degen, B., Caron, H., Lemes, M.R., Margis, R., Salgueiro, F. and Lowe, A.J. (2005) Optimal sampling strategy for estimation of spatial genetic structure in tree populations. *Heredity*, 95(4) 281-289.

Chase, M.W. and Hills, H.H. (1991) Silica gel: An ideal material for field preservation of leaf samples for DNA studies. *Taxon*, 215-220.

Cottrell, J.E., Munro, R.C., Tabbener, H.E., Gillies, A.C.M., Forrest, G.I., Deans, J.D. and Lowe, A,J, (2002) Distribution of chloroplast DNA variation in British oaks (*Quercus robur* and *Q. petraea*): The influence of postglacial colonisation and human management. *Forest Ecology and Management*, 156(1) 181-195.

Cottrell, J.E., Munro, R.C., Tabbener, H.E., Milner, A.D., Forrest, G.I. and Lowe, A.J. (2003) Comparison of fine-scale genetic structure using nuclear microsatellites within two British oakwoods differing in population history. *Forest Ecology and Management*, 176(1) 287-303.

Couvet, D. (2002) Deleterious effects of restricted gene flow in fragmented populations. *Conservation Biology*, 16(2) 369-376.

Degen, B., Caron, H., Bandou, E., Maggia, L., Chevallier, M.H., Leveau, A. and Kremer, A. (2001) Fine-scale spatial genetic structure of eight tropical tree species as analysed by RAPDs. *Heredity*, 87(4) 497-507.

Double, M.C., Peakall, R., Beck, N.R. and Cockburn, A. (2005) Dispersal, philopatry and infidelity: Dissecting local genetic structure in superb fairy-wrens (*Malurs cyaneus*). *Evolution*, 59(3) 625-635.

Dumolin-Lapegue, S. and Demesure, B. (1997) Phylogeographic structure of white oaks throughout the European continent. *Genetics,* 146(4) 1475.

Eckert, C.G. (2002) *The loss of sex in clonal plants.* Springer.

Ellstrand, N.C. and Elam, D.R. (1993) Population genetic consequences of small population size: Implications for plant conservation. *Annual Review of Ecology and Systematics,* 24 217-242.

Ellstrand, N.C. and Schierenbeck, K.A. (2000) Hybridization as a stimulus for the evolution of invasiveness in plants? *Proceedings of the National Academy of Sciences of the United States of America,* 97(13) 7043-7050.

Epperson, B.K. (1995) Spatial distributions of genotypes under isolation by distance. *Genetics,* 140(4) 1431-1440.

Escudero, A., Iriondo, J.M. and Torres, M.E. (2003) Spatial analysis of genetic diversity as a tool for plant conservation. *Biological Conservation,* 113(3) 351-365.

Evanno, G., Regnaut, S. and Goudet, J. (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology,* 14(8) 2611-2602.

Excoffier, L., Smouse, P.E. and Quattro, J.M. (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics,* 131(2) 479-491.

Falush, D., Stephens, M. and Pritchard, J.K. (2003) Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics,* 164(4) 1567-1587.

Friar, E.A., Boose, D.L., Ladoux, T., Roalson, E.H. and Robichaux, R.H. (2001) Population structure in the endangered Mauna Loa silversword, *Argyroxiphium kauense* (Asteraceae), and its bearing on reintroduction. *Molecular Ecology,* 10(7) 1657-1663.

Fromm, M. and Hattemer, H.H. (2003) Inheritance of allozymes and hybridization in two European *Tilia* species. *Heredity,* 91(3) 337-344.

Gaafar, A.R., Al-Qurainy, F. and Khan, S. (2014) Assessment of genetic diversity in the endangered populations of *Breonadia salicina* (Rubiaceae) growing in the kingdom of Saudi Arabia using inter-simple sequence repeat markers. *BMC Genetics*, 15 109-014-0109-4.

Gil, L., Fuentes-Utrilla, P., Soto, Á., Cervera, M.T. and Collada, C. (2004) Phylogeography: English elm is a 2,000-year-old roman clone. *Nature*, 431(7012) 1053-1053.

Glenn, T.C. and Schable, N.A. (2005) Isolating Microsatellite DNA Loci. In: Elizabeth A. Zimmer and Eric H. Roalson (eds.) *Methods in Enzymology.* San Diego CA: Academic Press, 202-222.

Gonzalez-Rodriguez, A., Arias, D.M., Valencia, S. and Oyama, K. (2004) Morphological and RAPD analysis of hybridization between *Quercus affinis* and *Q. laurina* (Fagaceae), two Mexican red oaks. *American Journal of Botany*, 91(3) 401-409.

Goudet, J. (1995) FSTAT (version 1.2): A computer program to calculate F-statistics. *Journal of Heredity*, 86(6) 485-486.

Goudet, J. (2001) *FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from http://www.unil.ch/izea/softwares/fstat.html. Updated from Goudet (1995).*

Hamrick, J.L., Linhart, Y.B. and Mitton, J.B. (1979) Relationships between life history characteristics and electrophoretically detectable genetic variation in plants. *Annual Review of Ecology and Systematics*, 10 173-200.

Hamrick, J.L., Mitton, J.B. and Linhart, Y.B. (1981) Levels of genetic variation in trees: influence of life history characteristics. In: *Procedure Symposium on Isozymes of North American Forest Trees and Forest Insects. Technical Report*, 35-41.

Hamrick, J.L., Godt, M.J.W. and Sherman-Broyles, S.L. (1992) Factors influencing levels of genetic diversity in woody plant species. *New Forests*, 6 95-124.

Hansen, O.K., Thomsen, P. and Rasmussen, C.W. (2014) DNA markers provide insight about common lime in historical plantings – An example from the Royal Danish Gardens. *Urban Forestry & Urban Greening, 13 543-552*).

Harter, A.V., Gardner, K.A., Falush, D., Lentz, D.L., Bye, R.A. and Rieseberg, L.H. (2004) Origin of extant domesticated sunflowers in eastern north America. *Nature,* 430(6996) 201-205.

Hewitt, G.M. (1999) Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society,* 68(1) 87-112.

Heywood, J.S. (1991) Spatial analysis of genetic variation in plant populations. *Annual Review of Ecology and Systematics,* 335-355.

Hubisz, M.J., Falush, D., Sephens, M. and Pritchard, J.K. (2009) Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources,* 9(5) 1322-1332.

Jakobsson, M. and Rosenberg, N.A. (2007) CLUMPP: A cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics,* 23(14) 1801-1806.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P. and Drummond, A. (2012) Geneious basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28(12) 1647-1649.

Lepais, O., Petit, R.J., Guichoux, E., Lavabre, J.E., Alberto, F., Kremera, A. and Gerber, S. (2009) Species relative abundance and direction of introgression in oaks. *Molecular Ecology,* 18(10) 2228-2242.

Liesebach, H. and Sinko, Z. (2008) A contribution to the systematics of the genus *Tilia* with respect to some hybrids by RAPD analysis. *Dendrobiology*, 59 13-22.

Logan, S.A., Phuekvilai, P. and Wolff, K. (2015) Ancient woodlands in the limelight: Delineation and genetic structure of ancient woodland species *Tilia cordata* and *Tilia platyphyllos* (Tiliaceae) in the UK. *Tree Genetics & Genomes,* 11(3) 1-12.

Loveless, M.D. and Hamrick, J.L. (1984) Ecological determinants of genetic structure in plant populations, *Annual Review of Ecology and Systematics,* 15 65-95.

Lowe, A., Harris, S. and Ashton, P. (2004) *Ecological Genetics: Design, Analysis and Application.* Blackwell Publishing.

Mantel, N. (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research,* 27(2) 209-220.

Miranda, C., Urrestarazu, J., Santesteban, L.G., Royo, J.B. and Urbina, V. (2010) Genetic diversity and structure in a collection of ancient Spanish pear cultivars assessed by microsatellite markers. *Journal of the American Society for Horticultural Science,* 135(5) 428-437.

Neophytou, C., Aravanopoulos, F.A., Fink, S. and Dounavi, A. (2011) Interfertile oaks in an island environment. II. limited hybridization between *Quercus alnifolia* Poech and *Q. coccifera* L. in a mixed stand. *European Journal of Forest Research,* 130(4) 623-635.

Nybom, H. and Bartish, I.V. (2000) Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. *Perspectives in Plant Ecology, Evolution and Systematics,* 3(2) 93-114.

Ordnance Survey (2014a) *Boundary Line: High Water [Shape geospatial data], Coverage: Great Britain.* [map], 1:10000 Ordnance Survey (GB). Available from EDINA Digimap Ordnance Survey Service, <http://digimap.edina.ac.uk [Accessed 26/08/14].

Ordnance Survey (2014b) *Boundary Line: Parish [Shape geospatial data], Coverage: Great Britain.* [map], 1:10000 Ordnance Survey (GB). Available from EDINA Digimap Ordnance Survey Service, <http://digimap.edina.ac.uk [Accessed 26/08/14].

Ordnance Survey (2014c) *1:25 000 Raster [TIFF geospatial data], tiles: SU41, SU50, SU51, SU60, SU61, SU70, SU71.* [map], 1:25000 Ordnance Survey (GB). Available from

EDINA Digimap Ordnance Survey Service, <http://digimap.edina.ac.uk; [Accessed 27/08/14].

Ordnance Survey (2014d) *1:25 000 Raster [TIFF geospatial data], tiles: TF06, TF07, TF08, TF16, TF17, TF18.* [map], 1:25000 Ordnance Survey (GB). Available from EDINA Digimap Ordnance Survey Service, <http://digimap.edina.ac.uk; [Accessed 27/08/14].

Osborne, J.L., Clark, S.J., Morris, R.J., Williams, I.H., Riley, J.R., Smith, A.D. Reynolds, D.R. and Edwards, A.S. (1999) A landscape study of bumble bee foraging range and constancy, using harmonic radar. *Journal of Applied Ecology*, 36(4) 519-533.

Peakall, R., Ruibal, M. and Lindenmayer, D.B. (2003) Spatial Autocorrelation Analysis Offers New Insights into Gene Flow in the Australian Bush Rat, *Rattus fuscipes*. *Society for the Study of Evolution*. 57(5) 1182- 1195.

Peakall, R., Ebert, D., Cunningham, R. and Lindenmayer, D. (2006) Mark–recapture by genetic tagging reveals restricted movements by bush rats (*Rattus fuscipes*) in a fragmented landscape. *Journal of Zoology*, 268(2) 207-216.

Peakall, R. and Smouse, P.E. (2006) GENALEX 6: Genetic analysis in excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6(1) 288-295.

Peakall, R. and Smouse, P.E. (2012) GenAlEx 6.5: Genetic analysis in excel. population genetic software for teaching and research - an update. *Bioinformatics*, 28(19) 2537-2539.

Petit, R.J., Latouche-Hallé, C., Pemonge, M. and Kremer, A. (2002) Chloroplast DNA variation of oaks in France and the influence of forest fragmentation on genetic diversity. *Forest Ecology and Management*, 156(1) 115-129.

Phuekvilai, P. (2014) *Relicts, refugia and reticulation: a study of population history, hybrids and phylogeny in the long-lived flowering tree genus Tilia.* PhD Newcastle University.

Phuekvilai, P. and Wolff, K. (2013) Characterization of microsatellite loci in *Tlia platyphyllos* (Malvaceae) and cross-amplification in related species. *Applications in Plant Sciences*, 1(4) 1200386.

Pigott, C.D. (1969) The status of *Tilia cordata* and *T. platyphyllos* on the Derbyshire limestone. *Journal of Ecology*, 57(2) 491-504.

Pigott, C.D. and Huntley, J.P. (1980) Factors controlling the distribution of *Tilia cordata* at the northern limits of its geographical range. II history in North West England. *New Phytologist*, 84(1) 145-164.

Pigott, C.D. (1991) Biological flora of the British Isles. no. 174 *Tilia cordata* Miller. *Journal of Ecology*, 79(4) 1147-1207.

Pigott, D. (1992) The clones of common lime (*Tilia*×*vulgaris* Hayne) planted in England during the seventeenth and eighteenth centuries. *New Phytologist*, 121(3) 487-493.

Pigott, D. (2012) *Lime-trees and basswoods: a biographical monograph of the genus Tilia*. Cambridge University Press.

Pilot, M., Dąbrowski, M.J., Hayrapetyan, V., Yavruyan, E.G., Kopaliani, N., Tsingarska, E., Bujalska, B., Kamiński, S. and Bogdanowicz, W. (2014) Genetic variability of the grey wolf *Canis lupus* in the Caucasus in comparison with Europe and the Middle East: distinct or intermediary population? PLOS ONE, 9(4) e93828.

Pritchard, J.K., Stephens, M. and Donnelly, P. (2000) Inference of population structure using multilocus genotype data. *Genetics*, 155(2) 945-959.

Provan, J. and Bennett, K.D. (2008) Review: Phylogeographic insights into cryptic glacial refugia. *Trends in Ecology & Evolution,* 23 564-571.

Provan, J., Powell, W. and Hollingsworth, P.M. (2001) Chloroplast microsatellites: new tools for studies in plant ecology and evolution. *Trends in Ecology & Evolution*, 16(3) 142-147.

Rackham, O. (2008) *Ancient Woodlands: Modern Threats.* Blackwell Publishing.

Rajora, O.P., DeVerno, L., Mosseler, A. and Innes, D.J. (1998) Genetic diversity and population structure of disjunct Newfoundland and central Ontario populations of Eastern white pine (*Pinus strobus*). *Canadian Journal of Botany,* 76(3) 500-508.

Raymond, M. and Rousset, F. (1995) GENEPOP (version 1.2): Population genetics software for exact tests and ecumenism. *Journal of Heredity,* 86(3) 248.

Rendell, S. and Ennos, R.A. (2002) Chloroplast DNA diversity in *Calluna vulgaris* (heather) populations in Europe. *Molecular Ecology,* 11(1) 69-78.

Rhymer, J.M. and Simberloff, D. (1996) Extinction by Hybridization and Introgression. Annual Review of Ecology and Systematics, 27 83-109.

Rousset, F. (2008) GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources,* 8(1) 103-106.

Selkoe, K.A. and Toonen, R.J. (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters,* 9 615-629.

Sloop, C.M., Eberl, R. and Ayres, D.R. (2012) Genetic diversity and structure in the annual vernal pool endemic *Limnanthes vinculans* Ornduff (Limnanthaceae): Implications of breeding system and restoration practices. *Conservation Genetics,* 13(5) 1365-1379.

Smouse, P.E., Long, J.C. and Sokal, R.R. (1986) Multiple regression and correlation extensions of the Mantel test of matrix correspondence. *Systematic Zoology,* 627-632.

Smouse, P.E. and Peakall, R. (1999) Spatial autocorrelation analysis of individual multiallele and multilocus genetic structure. *Heredity,* 82(5) 561-573.

Soltis, P.S. and Soltis, D.E. (2009) The role of hybridization in plant speciation. *Annual Review of Plant Biology,* 60 561-588.

Sork, V.L. and Smouse, P.E. (2006) Genetic analysis of landscape connectivity in tree populations. *Landscape Ecology,* 21(6) 821.

Stacy, E.A., Johansen, J.B., Sakishima, T., Price, D.K. and Pillon, Y. (2014) Incipient radiation within the dominant Hawaiian tree *Metrosideros polymorpha*. *Heredity*,113 334-342.

Streiff, R., Labbe, T., Bacilieri, R., Steinkellner, H., Glössl, J. and Kremer, A. (1998) Within-population genetic structure in *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. assessed with isozymes and microsatellites. *Molecular Ecology*, 7(3) 317-328.

Streiff, Ducousso, Lexer, Steinkellner, Gloessl, Kremer, Streiff, Ducousso, Lexer, Steinkellner, Gloessl, Kremer, Steiff, R., Ducousso, A., Lexer, C., Steinkellner, H., Gloessl, J. and Kremer, A. (1999) Pollen dispersal inferred from paternity analysis in a mixed oak stand of *Quercus robur* L. and *Q. petraea* (Matt.) Liebl. *Molecular Ecology*, 8(5) 831-841.

Sutherland, B.G., Belaja, A., Nier, S., Cottrell, J.E., Vaughan, S.P., Hubert, J. and Russell, K. (2010) Molecular biodiversity and population structure in common ash (*Fraxinus excelsior* L.) in Britain: Implications for conservation. *Molecular Ecology*, 19(11) 2196-2211.

Svejgaard Jensen, J. (2003) *Technical Guidelines for genetic conservation and use for lime (Tilia* spp*).* International Plant Genetic Resources Institute, Rome, Italy.: EUFORGEN.

Tollefsrud, M.M., Kissling, R., Gugerli, F., Johnsen, Ø., Skrøppa, T., Cheddadi, R., Van der Knaap, W., Latałowa, M., Terhurne-Berson, R. and Litt, T. (2008) Genetic consequences of glacial survival and postglacial colonization in Norway spruce: combined analysis of mitochondrial DNA and fossil pollen. *Molecular Ecology*, 17(18) 4134-4150.

Vaha, J. and Primmer, C.R. (2006) Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. *Molecular Ecology*, 15(1) 63-72.

Valbuena-Carabana, M., Gonzales-Martinez, S.C., Hardy, O.J. and Gil, L. (2007) Fine-scale spatial genetic structure in mixed oak stands with different levels of hybridization. *Molecular Ecology,* 16(6) 1207-1219.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M. and Shipley, P. (2004) Micro-checker: Software for identifying and correcting genotyping emrors in microsatellite data. *Molecular Ecology Notes,* 4(3) 535-538.

Van Rossum, F. and Triest, L. (2006) Fine-scale genetic structure of the common *Primula elatior* (Primulaceae) at an early stage of population fragmentation. *American Journal of Botany,* 93(9) 1281-1288.

Vaughan, S.P., Cottrell, J.E., Moodley, D.J., Connolly, T. and Russell, K. (2007a) Distribution and fine-scale spatial-genetic structure in British wild cherry (*Prunus avium* L.). *Heredity,* 98(5) 274-283.

Vaughan, S.P., Cottrell, J.E., Moodley, D.J., Connolly, T. and Russell, K. (2007b) Clonal structure and recruitment in British wild cherry (*Prunus avium* L.). *Forest Ecology and Management,* 242 419-430.

Weir, B.S. and Cockerham, C.C. (1984) Estimating F-Statistics for the analysis of population structure. *Evolution,* 38(6). 1358-1370.

Whitham, T.G. and Maschinski, J. (1996) Current hybrid policy and the importance of hybrid plants in conservation. In: *Southwestern Rare and Endangered Plants: Proceedings of the Second Conference. General Technical Report RM-283. US Forest Service, Rocky Mountain Forest and Range Experiment Station, Ft. Collins, Colorado*, 103-112.

Wicksell, U. and Christensen, K.I. (1999) Hybridization among *Tlia cordata* and *T. platyphyllos* (Tiliaceae) in Denmark. *Nordic Journal of Botany,* 19(6) 673-684.

Wilk, J.A., Kramer, A.T. and Ashley, M.V. (2009) High variation in clonal vs. sexual reproduction in populations of the wild strawberry, *Fragaria virginiana* (Rosaceae). *Annals of Botany,* 104(7) 1413-1419.

Woodlands Trust (2003) *Old Wood, Skellingthorpe management Plan 4641. Plan Period 2003 to 2008.* Woodlands Trust.

Wu, F.Q., Shen, S.K., Zhang, X.J., Wang, Y.H. and Sun, W.B. (2014) Genetic diversity and population structure of an extremely endangered species: The world's largest rhododendron. *AoB PLANTS,* 7. plu082.

Young, A., Boyle, T. and Brown, T. (1996) The population genetic consequences of habitat fragmentation for plants. *Trends in Ecology and Evolution,* 11(10) 413-418.

Zalapa, J.E., Brunet, J. and Guries, R.P. (2010) The extent of hybridization and its impact on the genetic diversity and population structure of an invasive tree, *Ulmus pumila* (Ulmaceae). *Evolutionary Applications,* 3(2) 157-168.

Zhou, H. and Chen, J. (2010) Spatial genetic structure in an understorey dioecious fig species: the roles of seed rain, seed and pollen mediated gene flow, and local selection *Journal of Ecology*, 98(5) 1168-1177.

Zhao, Y., Vrieling, K., Liao, H., Xiao, M., Zhu, Y., Rong, J., Zhang, W., Wang, Y., Yang, J. and Chen, J. (2013) Are habitat fragmentation, local adaptation and isolation by distance driving population divergence in wild rice *Oryza rufipogon*? *Molecular Ecology,* 22(22) 5531-5547.

CHAPTER FIVE

Tissue Culture

5.1 Tissue Culture Introduction

Tilia cordata Mill. is an indicator of ancient woodlands and as such is an important part of the ecosystem within the Bardney Limewoods. With efforts being made to extend the area of woodland and to increase connectivity between the woods, planting stock of local provenance is preferred to preserve the genetic character of the lime trees in this area. However, because of the concern about introducing stock from unknown sources, *T. cordata* has not been currently planted in any of the new woodland areas. Using *T. cordata* derived from seeds obtained locally would be one way to provide planting stock but viable seed from *T. cordata* within the Bardney Limewoods does not occur frequently and its viability appears variable, possibly being dependant on summer temperature (Pigott and Huntley, 1981; Kärkönen, 2000). Tissue culture could, if successful, offer the potential to produce trees of known provenance with rejuvenated rooting and flowering characteristics compared with the original trees (Webster and Jones, 1989; Jones *et al*., 1996; Smulders and De Klerk, 2011; Ballester and Vieitez, 2012). The cryopreservation of explants produced using tissue culture techniques may also offer the possibility of maintaining the germplasm of important *T. cordata* individual trees and populations over long periods of time (Quain *et al*., 2009; Uchendu *et al*., 2013; Smith, 2000). Information from the genetic study of *T. cordata* within the Bardney Limewoods will allow management decisions to be made that preserve the current diversity of the trees and allow trees to be selected for replanting schemes.

The main factors that influence successful tissue culture are considered to be (Murashige, 1974): -

- Explant selection
- Aseptic tissue sterilisation
- Tissue culture medium
- Plant growth regulators

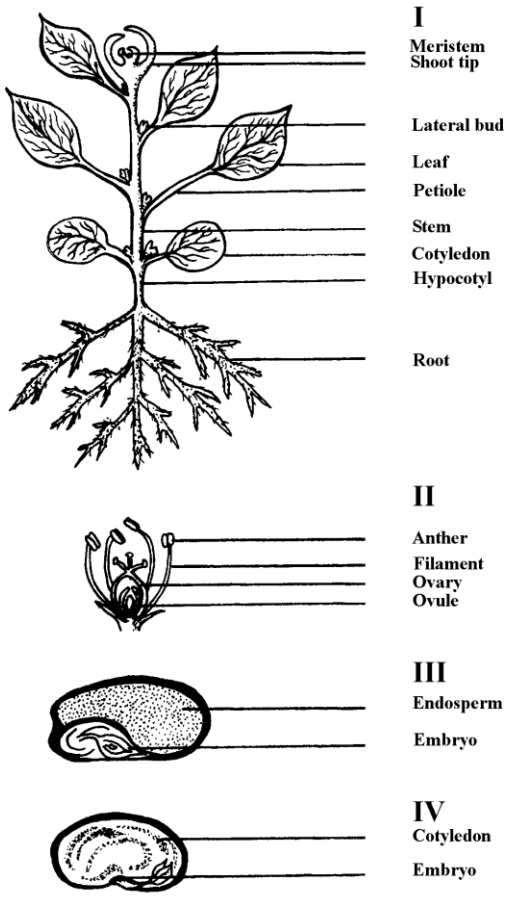
Careful explant selection and preparation is a prerequisite to the successful outcome of the tissue culture process and an initial choice of poor quality plant material can seriously compromise the whole process.

5.1.1 Explant Selection

Explants can potentially be derived from any part of the plant due to the totipotent nature of plant cells (figure 5.1). In reality, however, the choice of which tissue type to use for the explant is influenced by the required response from the cell culture. For somatic embryogenesis the explants are generally taken from cotyledon, hypocotyl, stem, leaf or embryo tissue whereas for the production of clonal plants microshoots, lateral or terminal buds or shoots are usually selected. Deriving clonal plants via tissue culture can, however, be problematic as the process itself has been found to produce somaclonal variation in cultured plants (Larkin and Scowcroft, 1981; Cassells and Curry, 2001; Bairu *et al*., 2011; Neelakandan and Wang, 2012) including woody plant species such as *Populus* (Lester and Berbee, 1977; Fry *et al*., 1997) and *Ulmus* (Lineberger *et al*., 1990; Domir and Schreiber, 1993). Factors such as genetic predisposition and the use of potentially mutagenic plant growth regulators such as 2,4-dichlorophenoxyacetic acid (2,4D), α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) to induce callus

formation are thought to increase the likelihood of genetic mutations in plantlets such as changes in ploidy, chromosomal rearrangement and single gene mutations (Phillips *et al*., 1994; Skirvin *et al*., 1994; Fry *et al*., 1997; McCown, 2000; Cassells and Curry, 2001; Ďurkovič and Mišalová, 2008) or more temporary epigenetic effects (Smulders and De Klerk, 2011). Rooted plantlets from microshoots derived from explants taken from preformed shoots containing meristem tissue, i.e. axillary buds and shoot tips, or from hardwood cuttings are less likely to be affected by somaclonal variation (Ostry *et al*., 1994; Fry *et al*., 1997).

Figure 5.1 Potential sources of explant tissues (Smith, 2000). I) plant stem, II) flower, III) monocotyledonous seed, IV) dicotyledonous seed.



The physiological age of the plant and morphological age of the tissue that is to be the source of the explant can also affect the final outcome of the tissue culture. Explant material from older plant sources has been found to be less responsive to culture (Lester and Berbee, 1977; Bonga, 1987; Von Aderkas and Bonga, 2000; Bonga *et al*., 2010). Younger tissue from mature trees, such as that derived from epicormic growth and basal sprouts in *Quercus robur* L. and *Q. rubra* L. (Sanchez *et al*., 1996), and *Castanea sativa* Mill. and *C. sativa* x *C. crenata* (Sanchez and Vieitez, 1991) have shown better response to tissue culture than that sourced from material sourced from the crown of the tree. This was also found with the *in vitro* propagation from 55-year-old *Tilia* platyphyllos Scop. trees, where Chalupa (2003) found that in culture "the rate of shoot-forming buds was dependant on plant age, genotype and bud position along shoot segments". Variation in successful tissue culture outcome has also been found to be affected by the season in which the explants are collected, with spring being the optimum time for tissue culture of buds from Douglas fir (Gupta and Durzan, 1987) and late July and August for the collection of seeds for somatic embryogenesis of *T. cordata* (Chalupa, 1990).

Kärkönen (2000) and Chalupa (1990) have previously produced somatic embryos of *T. cordata* from the culture of immature cotyledonary embryos. This method was not investigated in this current study as seeds of *T. cordata* were not observed in the Bardney Limewoods at the time of the trial.

5.1.2 Aseptic Tissue Sterilisation

The removal of fungal and bacterial contaminants from explant tissue is an essential step in the establishment of successful sterile plant tissue culture. A large proportion of the failures that occur in the production of viable plantlets occur as a result of contamination that is probably introduced to the culture through the explant or through the tissue culture process itself (e.g. tissue handling and preparation and medium preparation) (Leifert and Cassells, 2001).

To produce contamination-free explants, that will be responsive to culture, an effective process of tissue surface sterilisation that is not excessively damaging to the plant tissue needs to be established for the plant being studied. Two widely used biocides, sodium hypochlorite (NaClO) and sodium dichloroisocyanurate (NaDCC), disassociate to produce hyperchlorous acid (HOCl) in solution (Clasen and Edmondson, 2006). NaDCC is less phytotoxic than NaClO and does not need to be rinsed from plant tissue (Niedz and Bausher, 2002). Plant Preservative Mixture (PPM^{TM}) (Guri and Patel, 1998), a commercial preparation of two industrial isothiazolone biocides (methylchloroisothiazolinone and methylisothiazolinone), has been found to be effective against fungal and bacterial contamination when used prophylactically within the tissue culture medium (Niedz, 1998). Mercuric chloride ($HgCl_2$) can also be used for difficult to sterilise explants tissue but is highly toxic and corrosive to plant tissue (Chalupa, 1984; 2003).

5.1.3 Tissue Culture Medium

The composition of the medium used for tissue culture establishment and maintenance needs to be varied according to the type of plant being cultured and the type of growth response required. Nutrient media are designed to provide a mixture of inorganic salts and organic compounds to support the growth of the plant tissue during culture. Inorganic salts supply macroelements (e.g. nitrates and phosphates), microelements (e.g. cobalt and copper) and an iron source to the culture. The organic compounds that may be included in the medium are plant growth regulators, vitamins, hexitols (e.g. myo-inositol), a carbohydrate source (e.g. sucrose) and a gelling agent, usually agar.

With the widely-used Murashige and Skoog inorganic formulation (MS) (Murashige and Skoog, 1962) the inorganic salts, which replaced the previously-used organic supplements such as coconut milk and yeast extract, provide high levels of nitrate, potassium and ammonium to ensure that nutrients are present in sufficient quantities to sustain cell growth during culture. For woody plants, which are often less tolerant of the high salt and chloride levels found in MS, Lloyd and McCown (1980) developed woody plant medium (WPM). (Appendix 3)

Although plants can produce their own compounds which are essential for plant metabolism, vitamins such as thiamine (Vit. B_1), nicotinic acid (niacin) and pyroxidine (Vit. B_6), as well as myo-inositol, have been found to be necessary to promote cell growth during the culture of some plants. MS medium contains all three vitamins, as well as myo-inositol (Murashige and Skoog, 1962), but only thiamine and myo-inositol were found to be required for tobacco culture (Linsmaier and Skoog, 1965).

A carbohydrate source, usually sucrose at between 2 and 5%, is generally included in the medium as plants are not normally photosynthetically active during culture and require an

energy and carbon source. Agar is normally used as a gelling agent to set the medium; this provides a support for the plant tissue that also enables nutrients to diffuse through the medium to the plant cells.

Successful *in vitro* propagation of axillary shoots from *T. platyphyllos* and *T. cordata* has been achieved by Chalupa (1984; 2003) using modified MS medium.

5.1.4 Plant Growth Regulators

Plant growth regulators, or phytohormones, are important signalling molecules that influence the growth and development of plants (Moubayidin *et al*., 2009). Auxins, cytokinins, gibberellins and ethylene are phytohormones that affect plant growth and can be used to manipulate the tissue produced in culture. Auxins (e.g. indol-3-butyric acid (IBA), naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D)) influence cell enlargement and root initiation. Auxins also influence adventitious bud formation and suppress the development of lateral buds. Cytokinins (e.g. Kinetin, 6-benzyl-aminopurine (BAP) and N-benzyl-9-(2-tetrahydropyranyl) adenine (BPA)) promote cell division, shoot proliferation and shoot morphogenesis. When used together in culture the molar ratio, strength and stability of the plant growth regulators influences the ontogenic effect observed in the explant. A higher proportion of auxin generally produces roots whereas excess cytokinin tends to produce shoots. Undifferentiated callus tissue occurs when the proportions of cytokinin and auxin are approximately equal (Skoog and Miller, 1957).

5.1.5 Aim

The source, preparation and sterilisation of explants and choice of plant growth regulators were investigated in order to determine the feasibility of establishing microshoot cultures of *T. cordata* derived from known trees within the Bardney Limewoods

Within the Bardney Limewoods National Nature Reserve new areas of woodland are being created to both increase the total area of woodland within the reserve and increase connectivity between the wooded areas. At present, because of concerns about introducing trees of unknown genetic provenance and the possibility of introducing diseased stock from unknown sources, *T. cordata* has not been included in any new planting. Rooted plantlets, with possibly rejuvenated rooting and flowering characteristics compared with the original ancient trees, which resulted from successful culture of tissue from selected *T. cordata* trees could possibly be used in future planned planting schemes.

5.2 Tissue Culture Materials and Methods

All chemicals for the preparation of media were of analytical grade and obtained from Sigma-Aldrich Ltd. Plant Preservative Mixture™ (PPM™) (Guri and Patel, 1998) was obtained from Plant Cell Technology (Washington D.C). All tissue culture media and glassware were sterilised by autoclaving at 15 psi. (121ºC) for 15 minutes. All aseptic transfers and subculturing were carried out in a laminar flow cabinet and instruments used were sterilised by flaming with ethanol or autoclaving. The sterile *T. cordata* leaf and bud tissue was aseptically cut into sections and placed onto tissue culture medium containing plant growth regulators. The explants were kept at between 20 and 24ºC in artificial daylight with a 16-hour photoperiod as described by Chalupa (1990) and inspected at regular intervals for growth of fungal or bacterial contamination. Infected explants were removed from the trials and destroyed by autoclaving at 30 psi. (132ºC) for 20 minutes. To estimate the significance of the results statistical analysis was carried out for the dependent means of pairs of results using two sample t-test between percents (using StatPac for Windows 15.1.16 (available from www.statpac.com)) and for the comparison of methods of sterilisation and response to plant growth regulators Pearson's chi-square test was calculated using Excel.

5.2.1 Explant Selection, Preparation and Sterilisation

Stems of *T. cordata*, 40 to 50 cm long with 8 to 10 buds, were collected from 108 trees from 13 woods in the study area to give a total of 414 explants (chapter 4). Tissue used for micropropagation was either leaf or bud tissue taken from either old coppiced trees or from old stools that had recently been coppiced. Material used for culture was either: -

- Leaf and bud material taken from the trees and used immediately after collection for tissue culture.
- Stems collected in April and May, shortly before the leaf buds would open naturally. These stems were kept in water in clean laboratory conditions the leaves allowed to open and shoots to develop. The new leaves and shoots were removed from the stems and sterilised for use as tissue culture explants.
- Leafy stems collected after May. These were kept in water in clean laboratory conditions and the old leaves removed to facilitate the development of the axial buds. These new leaves were allowed to open and new shoots develop. The new leaves and shoots were removed from the stems and sterilised for use as tissue culture explants.

The sterilisation of the explants was carried out with the following possible stages: -

- Prewash
- Sterilisation
- Sterile deionised water (DI water) rinse
- Inclusion of a biocide within the tissue culture medium

The *T. cordata* tissue was sterilised and rinsed as shown in table 5.1 and was

aseptically cut into sections containing one bud or leaf and placed initially onto MS tissue culture medium without plant growth regulators (appendix 3). The explants were kept at between 20 and 24ºC in artificial daylight with a 16-hour photoperiod and were inspected at regular intervals for growth or contamination. Results from these experiments were used to develop a sterilisation protocol for the subsequent tissue culture trials. Uncontaminated explants were recultured onto fresh medium containing appropriate plant growth regulators.

Table 5.1 Initial development of sterilisation protocols.

Treatment		1	2	3	4	5	6	7
Pre-treatment (prewash)		x	EthOH	Det	Det + EthOH	x	x	x
Sterilisation	concentration	20%	20%	20%	20%	20%	20%	20%
	Minutes	15	15	2x20	10	20	10	10
Rinse	No. of rinses	5	5	5	5	3	3	3
	Min/rinse	20	15	20	10	20	10	10
Sterilisation agent in medium		x	x	x	x	x	x	PPM™

Notes

Pretreatment

EthOH	70% ethanol rinse for 5 minutes
Det	Water rinse with 0.05% Teepol® detergent for 15 minutes.
Det+EthOH	Water rinse with 0.05% Teepol® detergent for 15 minutes before 1minute in 70% ethanol.

Sterilisation Sterilisation using commercial bleach solution at given concentration (%) and for given time (minutes).

- Bleach - Domestos Thick Bleach®, Unilever, <5% sodium hypochlorite, <5% non-ionic surfactants.

Rinse Number of rinses in sterile DI water. Time given for each rinse (minutes /rinse).

PPM™ 0.05% Isothiazolone biocide added to medium before pouring (Plant Preservation Medium).

x No treatment

5.2.2 The comparison of two chlorine-based sterilisation solutions

A total of 152 newly emerged shoots with axial buds and small leaves were removed from *T. cordata* stems and sterilised as shown in table 5.2. Shoots taken from only one tree in Goslings Corner Wood collected at the same time were used to ensure that similar levels of contamination occurred in all samples. Two chlorine-based solutions (sodium dichloroisocyanurate (NaDCC) and Domestos® bleach solution containing sodium hypochlorite (NaClO)) were compared. After sterilisation the explants were placed on MS medium, initially with no plant growth regulators added, subsequent reculture of any successful explants was onto MS medium containing 0.5mg/l NAA and 0.5mg/l BAP. The explants were examined visually for contamination after 34 days. The number of uncontaminated explants was determined following the sterilisation of the plant tissue with either high or low concentrations of NaDCC solutions or a 20 % bleach solution. For each of the sterilisation regimes sterilisation times were also varied. All solutions were made up with sterile DI water. The effect of including detergent in the sterilisation solution and the effect of rinsing on the survival of the tissue was also compared for the two sterilisation solutions.

Table 5.2 Method development plan for tissue culture explant sterilisation.

Treatment	1	2	3	4	5	6	7	8
Sterilisation	NaDCC	NaDCC	NaDCC	NaDCC	NaDCC	NaDCC	NaDCC	NaDCC
Concentration %	1	1	1	0.5	0.5	0.5	1	1
Time, minutes	20	30	40	20	30	40	30	30
Detergent	0	0	0	0	0	0	✓	0
No. of Rinses	2	2	2	2	2	2	2	0

Treatment	9	10	11	12	13	14	15	16
Sterilisation	NaDCC	NaDCC	NaDCC	NaDCC	Bleach	Bleach	Bleach	Bleach
Concentration %	1	0.5	0.5	0.5	20	20	20	20
Time, minutes	30	30	30	30	20	30	40	20
Detergent	✓	✓	0	✓	✓	✓	✓	✓
No. of Rinses	0	2	0	0	2	2	2	0

Treatment	17	18	19	20	21	22	23	24
Sterilisation	NaDCC	NaDCC	NaDCC	NaDCC	NaDCC	NaDCC	NaDCC	NaDCC
Concentration %	0.03	0.03	0.03	0.03	0.01	0.01	0.01	0.01
Time, hrs	48	48	48	48	48	48	48	48
Detergent	✓	✓	0	0	✓	✓	0	0
No. of Rinses	2	0	2	0	2	0	2	0

Notes

Sterilisation

- NaDCC - Sodium dichloroisocyanate
- Bleach - Domestos Thick Bleach®, Unilever, <5% sodium hypochlorite, <5% non-ionic surfactants.

Detergent Detergent added to sterilisation solution. 0.05% Teepol® added to sterilisation solution. Detergent was not added to bleach as this already contains surfactants.

No. of Rinses Number of 20 minute rinses in sterile DI water.

5.2.3 Tissue Culture Medium and Plant Growth Regulators

T. cordata samples were collected from 72 trees in the Bardney Limewoods during the months of April and May and leaf buds were allowed to open in clean laboratory conditions (chapter 4). A total number of 597 explants were cultured but as not all the buds opened together and three separate trials were started as sufficient samples became ready. The previously developed sterilisation protocol was used for all explants. Before sterilisation the leaf and axial bud sections were pre-treated with a 15 minute 0.05% Teepol® wash followed by a 1minute dip in 70% ethanol. Leaf and stem sections were surface sterilised for 20 minutes with a 20% Domestos® solution and rinsed 3 times in sterile DI water. The prepared explants were placed onto Murashige and Skoog (MS) tissue culture medium (appendix 3) containing plant growth regulators. The auxin, naphthaleneacetic acid (NAA), and the cytokinin, 6-benzyl-aminopurine (BAP), were used either individually or in combination to promote root, stem or callus growth in the explants (table 5.3). The explants were monitored over periods of up to 12 months and recultured onto fresh medium at regular intervals if not contaminated.

Table 5.3 Plant growth regulator combinations used to stimulate root, callus or shoot development in *T. cordata* tissue culture.

Auxin, naphthaleneacetic acid (NAA) mg/l	Cytokinin, 6-benzyl-aminopurine (BAP) mg/l					
	0	0.1	0.5	2.5	5.0	10.0
0	✓			✓	✓	✓
0.1		✓				
0.5	✓		✓			
1.0	✓					

5.3 Tissue Culture Results

5.3.1 Explant Selection, Preparation and Sterilisation

The explants from the initial experiments were inspected at regular intervals and the number of uncontaminated explants determined after 10 days in culture. The results from these trials were used to determine the most appropriate source of explants for subsequent studies and are summarised in table 5.4.

From the culture of axial buds and leaf tissue from the 133 samples taken directly from the woods contamination was visible on a large number of explants, with only 6.8 % of the samples being uncontaminated after 10 days of culture; this was significantly worse than the result from the 281 explants taken from leaf and bud tissue that had opened under clean indoor conditions before sterilisation where 31.7 % were still uncontaminated after 10 days of culture ($t_{df\ 412} = 5.57$, $p < 0.001$).

An application of the PPM™ biocide to the medium did not appear to be effective in reducing contamination when used to sterilise new tissue. When PPM™ was included in the medium 34.2% of explants were uncontaminated compared with 28.9% uncontaminated when PPM™ was not included ($t_{df\ 87} = 0.53$, $p = 0.60$). Pre-treating the plant tissue obtained from shoots allowed to open in laboratory conditions, by washing with detergent solution followed by an ethanol dip before sterilisation, only increased the total percentage of uncontaminated samples from 20.6% to 21.0%; this was not significant ($t_{df\ 234} = 0.061$, $p = 0.95$).

Table 5.4 Summary of results comparing explant source for all sterilisation methods. All plants sterilised in 20% bleach (Domestos®) solution and cultured onto MS medium with no plant growth regulators.

	Explants uncontaminated after 10 days	
	Tissue taken directly from the	New leaves allowed to emerge in
	%	%
All methods	6.8	31.7
Biocide in medium	Not compared	34.2
No biocide	Not compared	28.9
Pre-treatment	4.3	46.7
No pre-treatment	10.8	26.7

As a result of this initial study all subsequent tissue used for culture was derived from leaf or axial buds taken from shoots that had emerged under laboratory conditions. Even though pre-sterilisation treatment did not significantly reduce explant contamination a detergent wash followed by a 70% ethanol dip was included in the preparation of the explants.

5.3.2 The Comparison of Two Chlorine-Based Sterilisation Solutions

All the tissue for these trials was new leaf material taken from one tree in Goslings Corner Wood. The results (table 5.5) show that after 34 days at least 50% of the explants were uncontaminated for all methods and all times of sterilisation, except 0.5 % NaDCC, 20 minutes, where only 25% of explants were uncontaminated and a Chi-square test found no significant relationship between any of sterilisation procedures, $\chi^2_{df\ 10} = 1.26$, $p = 0.49$.

However, not all of the uncontaminated explants could be recultured, with many becoming brown as a result of tissue damage during the sterilisation process (fig. 5.2). Only with sterilisation for 20 minutes in 20% bleach solution was there a 100% yield of uncontaminated explants that were suitable for reculture. A Chi-square test suggests that significant differences between the sterilisation methods does occur if the suitability of explants to be recultured is also considered, $\chi^2_{df10} = 7.02$, $p > 0.001$. Longer periods of sterilisation in bleach gave progressively lower yields. Although sterilisation with NaDCC gave overall yields of uncontaminated explants between 50 and 75% for all concentrations, the yield suitable for reculture was worse for higher concentrations, being zero for NaDCC 1% but over 50% for 0.01% and 0.03% NaDCC.

Table 5.5 The effects of concentration and time of sterilisation on the percentage of uncontaminated explants observed after 34 days of culture. Also shown is the percentage of undamaged explants suitable for reculture after 34 days.

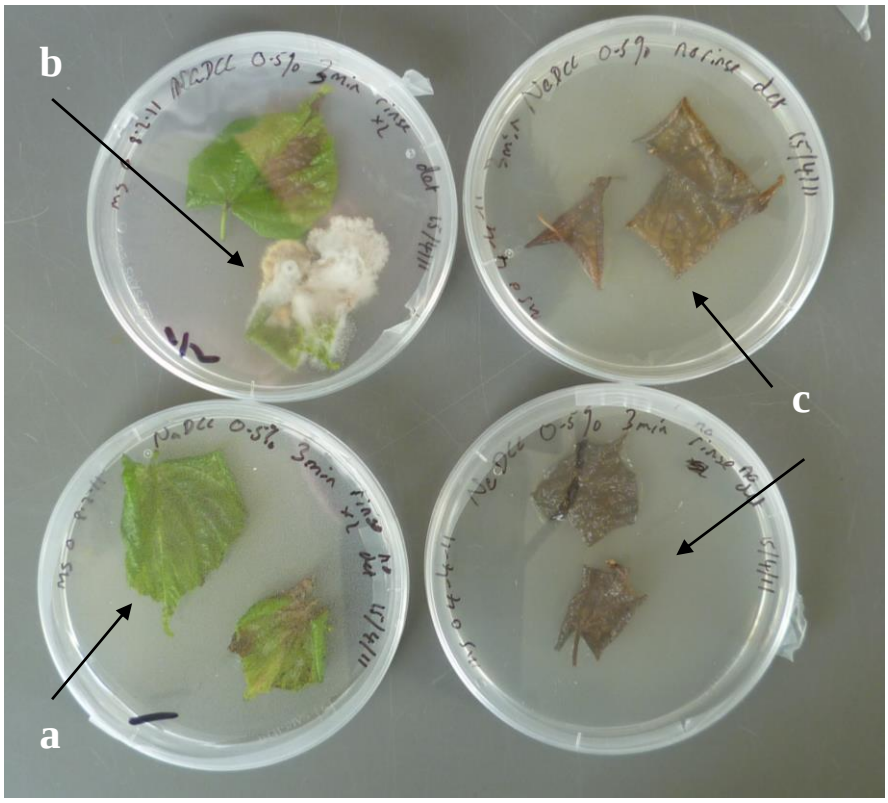
	Sterilisation Time	No. of explants	% Un-contaminated	% Suitable for reculture
Bleach 20% v/v	20min	8	100.0	100.0
	30min	8	100.0	50.0
	40min	8	50.0	12.5
	Overall	24	83.3	50.0
NaDCC 1% w/v	20min	16	75.0	0.0
	30min	16	50.0	0.0
	40min	16	75.0	0.0
	Overall	48	66.7	0.0
NaDCC 0.5% w/v	20min	16	25.0	12.5
	30min	16	75.0	12.5
	40min	16	50.0	0.0
	Overall	48	50.0	8.3
NaDCC 0.03% w/v	48hrs	16	75.0	62.5
NaDCC 0.01% w/v	48hrs	16	75.0	50.0

Figure 5.2 Cultures after 34 days.

a.) Uncontaminated explants.

b.) Contamination of leaf explants.

c.) Brown necrotic tissue as a result of aggressive sterilisation.



Of the 152 explants sterilised 100 appeared to be free of contamination after 34 days in culture but, as a result of tissue damage, only 34 of these were suitable for reculture. After a further 58 days of culture, contamination and browning of the tissue became increasingly problematic and only six explants remained uncontaminated and green. Of these six explants, four had been sterilised using 20% bleach solution for 20 minutes, one with bleach for 30 minutes and one with 0.5% NaDCC for 30 minutes.

Including 0.05% detergent in the sterilisation solution, to facilitate the wetting of the plant tissues by the biocide, only improved the yield for 0.03% NaDCC. For all other sterilising solutions the number of uncontaminated explants decreased when detergent was added (table 5.6). The Domestos® Bleach solution could not be compared as 5% non-ionic surfactant is included in the commercial formulation. As before, not all the uncontaminated tissue was suitable for reculture after sterilisation as many explants appeared damaged by the sterilisation process. Only with 0.01% NaDCC (no detergent) were 100% of the explants recultured. Including detergent in the biocide resulted in a reduction in the number of uncontaminated explants, 54.6% overall compared with 75.0% uncontaminated when detergent was not added ($t_{df150} = 2.58$, $p = 0.011$). This difference did not, however, remain significant when the total number explants that could be recultured were considered (26.1% with detergent and 25% without detergent ($t_{df150} = 0.16$, $p = 0.87$)).

Table 5.6 The effect of the inclusion of detergent in the sterilising solution on the percentage of uncontaminated explants and explants suitable for reculture observed after 34 days of culture.

Sterilisation	Detergent in sterilising solution	No. of explants	% Un-contaminated	% Suitable for reculture
Bleach 20%	✓	24	83.3	54.2
	(in bleach)		na	na
NaDCC 1% w/v	✓	24	50.0	0.0
	x	24	83.3	0.0
NaDCC 0.5% w/v	✓	24	33.3	16.7
	x	24	66.7	16.7
NaDCC 0.03% w/v	✓	8	100.0	75.0
	x	8	50.0	50.0
NaDCC 0.01% w/v	✓	8	0.0	0.0
	x	8	100.0	100.0
Overall	✓	88	54.6	26.1
	x	64	75.0	25.0

Detergent used in solution, 0.05% Teepol®

Bleach (20%)	sterilisation time 20 minutes
NaDCC (1% and 0.5%)	sterilisation time 30 minutes
NaDCC (0.03% and 0.01%)	sterilisation time 48 hours

Rinsing of the tissue with sterile DI water after sterilisation, to minimise tissue damage, is a normal procedure when using aggressive sterilising solutions such as NaClO. However, NaDCC is less phytotoxic than NaClO and can be left on plant tissue after sterilisation and may provide some protection against contamination during culture. Rinsing only appeared more effective in producing surface sterilised explants for 0.03% NaDCC (100% uncontaminated with two rinses, 50% with no rinse) (table 5.7). For all other solutions removing the final rinse produced more uncontaminated explants. However, leaving sterilising solution on the explant surface was deleterious to the plant tissue as the number available overall for reculture was reduced from 36.8% with two rinses to 14.4% with no rinse ($t_{df150} = 150$, $p = 0.0019$). In subsequent experiments all the explants were rinsed after sterilisation solution was used for decontamination.

Table 5.7 The effect of rinsing the explants after sterilisation on the percentage of uncontaminated explants and explants suitable for reculture observed after 34 days.

Sterilisation	Two rinses after sterilisation	No. of explants	% Un-contaminated	% Suitable for reculture
Bleach 20% v/v	✓	12	66.7	66.7
	x	12	100.0	41.7
NaDCC 1% w/v		24	33.3	0.0
	x	24	100.0	0.0
NaDCC 0.5% w/v	✓	24	16.7	33.3
	x	24	83.3	0.0
NaDCC 0.03% w/v	✓	8	100.0	100.0
	x	8	50.0	25.0
NaDCC 0.01% w/v	✓	8	50.0	50.0
	x	8	50.0	50.0
Overall	✓	76	42.1	36.8
	x	76	84.2	14.4

Bleach (20%)	sterilisation time 20 minutes
NaDCC (1% and 0.5%)	sterilisation time 30 minutes
NaDCC (0.03% and 0.01%)	sterilisation time 48 hours

5.3.3 Tissue Culture Medium and Plant Growth Regulators

After approximately one month of culture the 597 tissue samples taken from 72 trees produced, in three separate trials, a total of 80 explants with some type of tissue growth. The remaining 517 samples showed signs of contamination and/or necrotic brown tissue and were not recultured. The plant growth regulators included in the medium helped to stimulate the production of shoots in 20 samples, roots in 28 samples and undifferentiated callus type growth in 32 samples (fig. 5.3).

Shoots were induced to grow with all plant growth regulator combinations but the maximum number of explants occurred on medium with no growth regulator, where 4.2% of the cultured explants produced shoots (fig. 5.4). Root growth was initiated on medium with no growth regulators and all media containing the auxin NAA, with a concentration of 1mg/l producing the maximum percentage of successful samples (13.8%). However, increasing amounts of the cytokinin BAP resulted in progressively lower percentages of root development, with >2.5mg/l BAP producing none. Undifferentiated callus tissue was observed with all growth regulators except 0.5mg/l NAA, the maximum number of explants with callus occurring with 2.5mg/l BAP (13.95%). Chi squared tests for independence showed non-significant differences between plant growth regulator regimes for shoot and callus growth (Shoots, $\chi 2df\ 7 = 0.06$, $p = 0.99$; callus, $\chi 2df\ 7 = 13.81$, $p = 0.05$). For root proliferation, however, a significant difference between plant growth regulators was identified ($\chi 2df\ 7 = 33.9$, $p > 0.001$)

Figure 5.3 The development of explants growing on MS medium containing plant growth regulators: -

a.) 0.5 mg/l BAP and 0.5 mg/l NAA initiating shoot growth.

b.) 1.0 mg/l NAA initiating root growth.

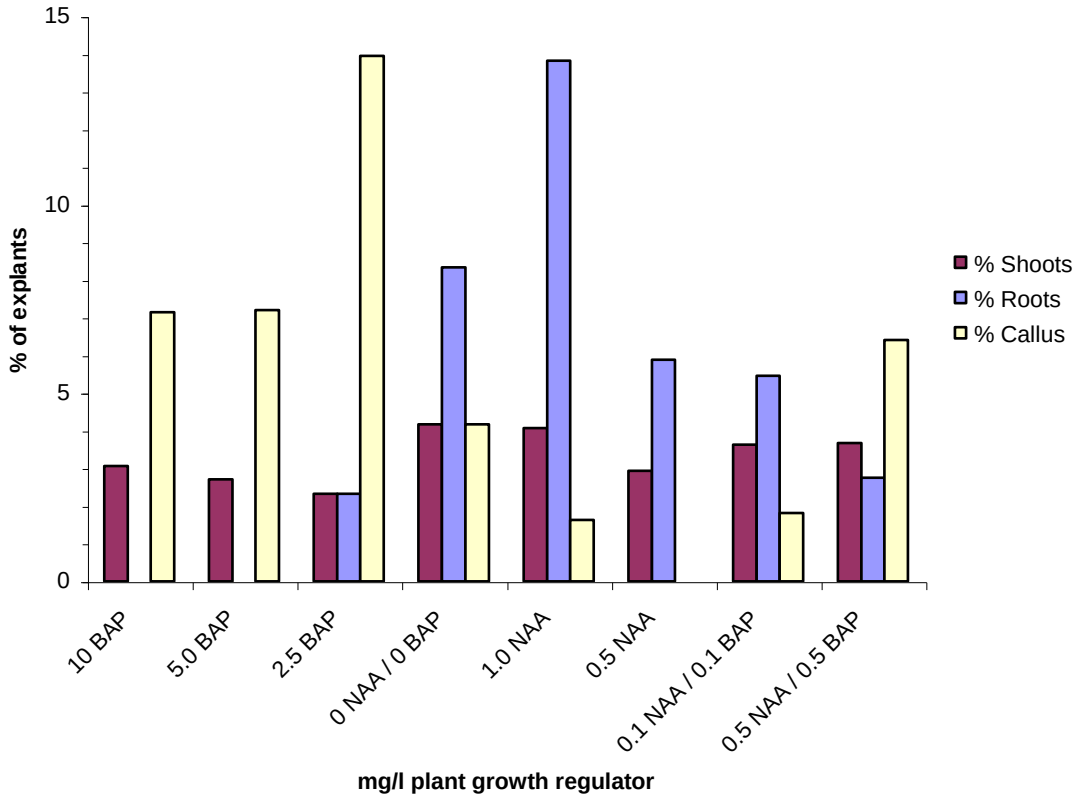
c.) 0.5 mg/l BAP and 0.5 mg/l NAA stimulating callus tissue formation.

a.)

b.)

c.)

Figure 5.4 The response of *T. cordata* explants, after 1month in culture on MS medium, to eight plant growth regulator regimes containing varying amounts of the plant growth regulators BAP and NAA (expressed in mg/l). The percentage of the number of explants producing shoots, roots or callus for each MS media is shown.



Explants that did not appear to be affected by bacterial and fungal contamination or tissue browning were recultured on the original medium at approximately monthly intervals. After five months of culture only 14 explants remained viable and had produced either a small number of roots or shoots, with no explant producing both (table 5.8). These 14 explants were all derived from two trees, Ivy Wood 4 (collected May 2006), and Goslings Corner Wood 4 (collected May 2006) (fig.5.5). After a further seven months of culture contamination continued to affect the explants and culture could not be continued.

Table 5.8 The effect of plant growth regulators on the growth of tissue culture samples after 5months in culture (initial sample number of trees = 72, number of tissue samples = 597).

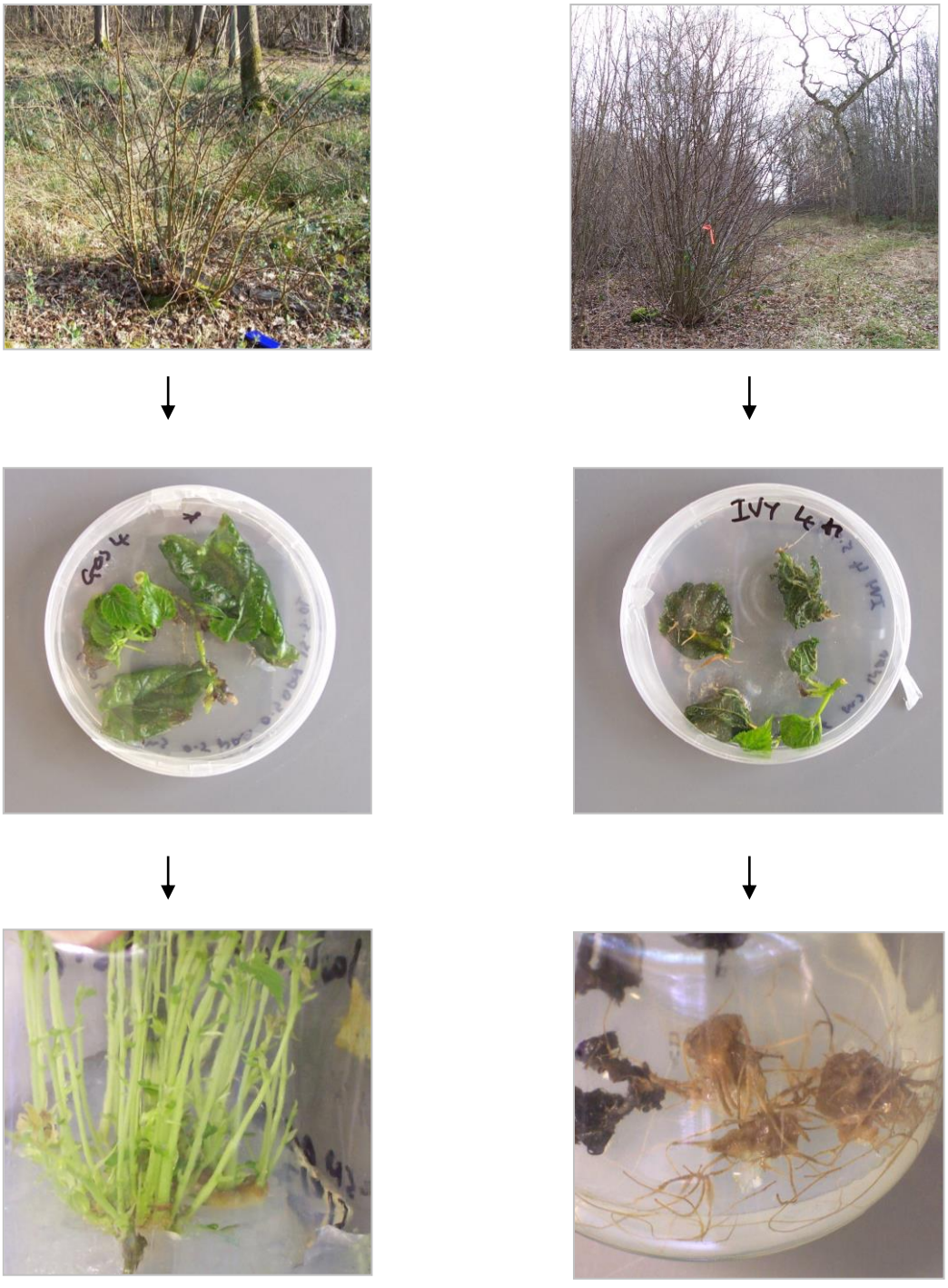
	Roots (no. of plants)	Shoots (no. of plants)	Callus (no. of plants)
NAA 1mg/l	9	2	-
BAP 0.5mg/l + NAA 0.5mg/l	2	1	-

Figure 5.5 Tissue culture progress for successful explants after 5 months culture.

a.) Goslings Corner (Tree 4) 0.5 mg/l NAA and 0.5mg/l BAP, producing shoots.

b.) Ivy Wood (Tree 4) 1.0 mg/l NAA, producing roots.

a.) **b.)**



5.4 Tissue Culture Discussion

Contamination of the prepared *T. cordata* explants, that could not be controlled by the initial surface sterilisation, was the major challenge in the establishment of successful tissue culture and was never resolved during this trial. From the initial results the treatment of the collected tissue prior to the preparation of the explants was found to be the most important factor in the production of surface-sterilised tissue that was free of fungal and bacterial contamination. Culturing leaves and axial buds from samples collected directly from the woods compared unfavourably with the results obtained using tissue derived from buds that had been allowed to open in a clean, dry environment after collection. Only 6.8% of opened samples from the woods were uncontaminated after 10 days of culture compared with 31.7% of those that had opened in the lab ($p<0.001$). Clean, uncontaminated cultures have generally been found to be easier to establish from explants derived from young plant tissue such as seedlings. This is thought to be because explants from older plants, especially those collected from the field, are more likely to contain endophytes that are difficult to eliminate (Pirttilä *et al*., 2003; Novotny, 2004). However, as seedlings were not available for this study and all explants were derived from old rootstocks it was not possible to determine whether this is the case for *T. cordata* from the Bardney Limewoods. Plants collected from outdoors are also more likely to harbour significant amounts of contamination compared with those grown under controlled conditions, such as in a greenhouse (Smith, 2000; Vijayan *et al*., 2011). In the micropropagation of mulberry (*Morus spp*.), Vijayan *et al.* (2011) reported that tissue taken from field grown trees was heavily contaminated with epiphytic and endophytic microorganisms, especially during the rainy season. It would seem likely, therefore, that the initial level of contamination on the surface of the tissue removed directly from the Bardney Limewoods was a factor in the resulting low number of sterile *T. cordata*

explants. Protecting new leaf and bud tissue from external sources of microorganism contamination, such as that carried by rainfall, by allowing buds to open in clean, dry conditions gave an improved outcome to the sterilisation process.

The comparison of sterilisation procedures using chlorine-based biocides was used to develop a method that could be used to produce surface-sterile explants. Although all the methods using the two biocides produced some explants that initially appeared contamination free, in many cases the sterilisation process itself damaged the explants to such an extent that they could not be recultured. For *T. cordata*, as with the sterilisation of *Hibiscus rosa-sinensis* L. by Dar *et al.* (2012), where immersion in a 1.1% sodium hypochlorite solution for 15 minutes was found optimum for internodal explant yield, increasing sterilisation solution concentrations and sterilisation times were both found to increase tissue damage and therefore reduce the number of explants that could be recultured.

Parkinson *et al.* (1996) compared the sterilisation of heavily contaminated *Spathiphyllum* petite with NaDCC and NaClO. When used at a concentration of 0.5%, NaDCC was found to be more effective than NaClO and gave significantly more uncontaminated shoots. Longer sterilisation with 0.03% NaDCC for between 24 and 48 hours was found to be as effective as 0.5% NaClO. Bausher and Niedz (1998) also found NaDCC to be more effective than NaClO in the sterilisation of explants, especially those derived from field grown citrus trees, and reported similar results to Parkinson *et al.* (1996). When comparing NaDCC and NaClO sterilising protocols for *T. cordata*, however, although no significant difference in the number of uncontaminated explants was found between any of the methods ($P=0.49$), significant differences between the NaDCC and NaClO

protocols were found if phytotoxicity was also considered ($P < 0.001$) with 20% Domestos® bleach solution (providing ≤ 1% NaClO) for 20 minutes gave the most effective way to reduce contamination producing 100% recultured explants.

As sterilisation with 20% Domestos® bleach solution resulted in 100% of explants being recultured, the bleach protocol was used in preference to any of the NaDCC based methods, for all subsequent explant preparation. Bleach was considered to be an effective and low cost decontaminant for *T. cordata* that could be easily rinsed away, thus preventing excessive explant tissue damage.

Endophytic bacteria and fungi, although making it difficult to produce clean cultures, may have a symbiotic role within the plant (Trotsenko *et al*., 2001) and have been found to be associated with almost all plants that have been examined (Carroll, 1988; Ryan *et al*., 2008). In *T. cordata* Doronina *et al.* (2004) isolated methylotropic bacteria that survived within the plant tissue over winter and produced biofilms on the leaf surface when the trees were growing. In Scots pine (*Pinus sylvestris* L.), bacteria and filamentous fungi were found to occur in buds, meristem and bud scales (Pirttilä *et al*., 2003; Pirttilä, 2011), with the metabolic activity of the microorganisms being reduced over the winter (Pirttilä *et al*., 2003; Pirttilä *et al*., 2005; Pirttilä, 2011). *In vivo* endophytic microorganisms may become pathogenic, outgrowing the explant on the tissue culture medium or they may remain latent. Latent contamination can affect the growth and rooting rates of the explant or become pathogenic at some later stage in the tissue culture process when the environmental conditions change (Leifert and Cassells, 2001). As contamination such as this may have affected the outcome of the *T. cordata* tissue cultures, strategies to combat the emergence of endophytic microorganisms from the surface-sterilised explants during

culture were investigated. Bausher and Niedz (1998) found that explants derived from field grown citrus trees were more likely to be contaminated after sterilisation than explants from greenhouse grown trees and achieved improved sterilisation rates by combining the use of NaDCC with the inclusion the antimicrobial agent PPM™ in the medium. However, for some plant species, PPM™ has been found to be phytotoxic, causing tissue death or slow shoot and tissue development (George and Tripepi, 2001). The addition of PPM™ to the medium, however, did not result in any significant reduction in contamination for *T. cordata* after 10 days of culture (35.3% uncontaminated with biocide in medium, 36.5% uncontaminated with no biocide in medium ($p = 0.60$)) and was not included in subsequent media preparations. Any deleterious effect of PPM™ was not observed but may have become significant if culture of the samples had been continued over an extended period of time.

Leaving NaDCC sterilising solution on the explants has been found, for some plant tissues, to also provide protection against contamination from endogenous bacteria arising from within the plant tissue during culture. Parkinson *et al.* (1996) found that 0.5% NaDCC, without rinsing, was effective at sterilising heavily contaminated *Spathiphyllum* petite. Although the antimicrobial action of both NaDCC and NaClO derives from the formation of hyperchlorous acid (HOCl) in solution (Clasen and Edmondson, 2006), NaDCC provides a more consistent level of HOCl and does not need to be rinsed from plant tissue after sterilisation as it is less toxic to plant tissue (Niedz and Bausher, 2002). For *T. cordata,* although there was a reduction in contaminated explants when the biocide was left on the tissue, significant tissue damage reduced the number of explants available for reculture (two rinses 36.8% recultured, no rinse 14.4% recultured ($P = 0.0019$)). The

omission of rinsing after sterilisation was not therefore incorporated into the final sterilisation protocol.

Although initial sterilisation of *T. cordata* had appeared successful, over a period of one year all explants gradually showed evidence either of contamination or became necrotic. Chalupa (1984; 2003), however, achieved successful sterilisation of oak and lime axillary buds using Mercuric chloride ($HgCl_2$) a more aggressive biocide than NaDCC or NaClO. Although $HgCl_2$ may have given a better sterilisation outcome in the long-term it is highly toxic and this method of sterilisation was not investigated.

For woody plants, microshoots, which can be used to produce rooted plantlets, can be derived from the establishment of stabilized shoot cultures from explants taken from the original tree. Plant growth regulators added to tissue culture media influence the growth and development of the explant. The response of the explants to the plant growth regulators in the medium is, however, moderated by the phytohormones that are already being made within the plant. Optimisation of the plant growth regulator regime for any plant tissue and required explant outcome is required as interaction between endogenous and exogenously applied plant growth regulators results in a range of responses from the explant tissue (Gaspar *et al*., 1996; Zulfiqar *et al*., 2009)

Chalupa (1987) propagated shoot tips and short nodal segments of seedling *T. cordata* and initiated shoot growth on modified MS media using 6-benzyl-aminopurine (BAP). Shoot growth occurred on media without growth regulators (1.3± 0.8 new shoots / explant) but increasing the concentration of BAP from 0.2 mg/l to 1.0 mg/l resulted in increasing number of shoots produced (4.7± 2.2 to 7.3 ± 2.5 new shoots). Higher

concentrations of BAP (4.0 mg/l) resulted in the production of shorter and fewer shoots (5.4 ± 2.3 new shoots). Chalupa (1984) also found that the inclusion of low levels of the auxins indole-3-butyric acid (IBA) or naphthaleneacetic acid (NAA) in the BAP supplemented media did not significantly affect the number or size of shoots produced. With the culture of axillary buds of 2-year-old *Tilia europaea x* L. on woody plant media (WPM), Sarvašová and Ďurkovič (2002) also found that shoots were produced from culture with either BAP on its own (0.2 to 3.0 mg/l) or together with NAA (0.1mg/l). However, in this case there was no significant difference between the results from any media combinations and the mean number of shoots per explant was 2.13 ± 1.09. For the explants taken from Bardney Limewoods trees it was also found that there was no significant difference observed between any of the plant growth regulator combinations for both callus and shoot growth (Callus, $p = 0.05$; shoots, $p = 0.99$). Shoot growth was observed across all BAP and NAA combinations as well as on medium that contained no plant growth regulators or contained either just NAA or BAP.

Chalupa (1984) induced the *T. cordata* microshoots to root using low concentrations of either NAA or IBA (0.1 – 0.5 mg/l) but found that the highest percentage of rooted shoots (70-95 %) occurred with IBA and NAA in combination (IBA, 0.3 mg/l and NAA, 0.1 mg/l). Sarvašová and Ďurkovič (2002) also rooted shoots with either IBA or NAA or a combination of IBA and NAA but found the greatest number of rooted shoots occurred on much higher levels of auxin than used by Chalupa (1984), 2.0mg/l NAA or 2.0 mg/l IBA. As microshoot cultures were not established in this study the conditions required to achieve rooted shoots was not determined. However, it was found that explant root growth could be initiated on a wide range of media including medium that included no plant growth regulators and on medium containing 2.5 mg/1 BAP. Only on media with

high levels of cytokinin (5mg/l BAP or 10mg/l BAP) did roots fail to develop. For root growth, significant differences ($p = >0.001$) between plant growth regulators combinations were seen and MS medium with 1.0 mg/l NAA was identified as giving the best result with 13.8% of the explants developing roots.

In conclusion, the development of a tissue culture protocol for *T. cordata* was variable. Difficulties encountered in obtaining sufficient contamination free material limited the opportunity to optimise a plant growth protocol that produces rooted plantlets. However, the most effective protocol developed involved the culture of newly emerged axial buds. These small buds were taken from plant tissue collected in April or May from which leaves had been allowed to open in clean laboratory conditions. The most effective subsequent sterilisation of the explants comprised a pre-treatment of a 15 minute, 0.05% Teepol® wash and a 1minute dip in 70% ethanol followed by sterilisation of the tissue for 20 minutes with a 20% Domestos® solution and three 20 minute rinses in sterile water. The initiation of microshoot cultures requires further development with investigation into the effects of different tissue culture media and plant growth regulators. However, shoots can be achieved using MS medium supplemented with 0.5 mg/1 NAA and 0.5 mg/l BAP; this was the plant growth regulator medium combination that had sustained the longest surviving cultures. Although NAA was able to initiate roots in some tissue, in order to optimise this protocol to produce rooted shoots further studies are also needed to investigate the plant growth regulators and tissue culture media needed to establish microshoot rooting.

Other strategies that might be used to improve the success of micropropagation from mature trees include: -

- Deriving explant tissue from shoots with juvenile characteristics from epicormic tissue taken from recently coppiced trees (Hernández *et al*., 2003).
- Deriving explants from immature embryos (Chalupa, 1990; Kärkönen, 2000).

Both of these sources of juvenile tissue are less likely to be heavily contaminated with microorganisms and may, therefore, be easier to sterilise and be more responsive to plant growth regulators.

Difficulty in achieving *in vitro* propagation of *T. cordata* from microshoots derived from axial buds may not, however, be just a result of poor tissue sterilisation. Woody plants are, in general, more difficult than herbaceous plants to culture with the tissue being recalcitrant and shoot, root and callus tissue slow to grow (Smith, 2000; Bairu and Kane, 2011). Tissue culture is also much less successful when the explant material is derived from mature plant material because the ability of the vegetative tissues to differentiate into leaves, roots and stems declines with age (Arnaud *et al*., 1993; Biroščíková *et al*., 2004; Ďurkovič and Mišalová, 2008). McCown (2000) suggests that this recalcitrance may be due, in part, to the genetic predisposition of the original "parent" tissue.

Although tissue culture holds the prospect of producing seedlings with rejuvenated characteristics (Ballester and Vieitez, 2012) it is not the only way to asexually propagate *T. cordata* within the Bardney Limewoods and other methods may be more successful and cost effective. In recent years, layered branches of *T. cordata* have been successfully rooted and, if successfully removed from the mother tree, could produce clonal planting

stock. The use of mist and dew point systems, successfully used for the acclimatisation of *U. procera* and other transgenic plants (Gartland *et al*., 2003; 2005), should also be investigated as a method that may facilitate the successful propagation of *T. cordata* from epicormic shoots for use in local planting schemes. Growing trees, however, from collected seed may provide a better yield of rooted trees than tissue culture and would increase the genetic variability of the local population. Although *T. cordata* is reported to have low fertility in Derbyshire and northern Britain (Pigott, 1969; Pigott and Huntley, 1981), seedlings have been found within the Bardney Limewoods and, in May 2012, many seedlings were found in Hatton Wood suggesting that this may now be a successful strategy.

5.5 References

Arnaud, Y., Franclet, A., Tranvan, H. and Jacques, M. (1993) Micropropagation and rejuvenation of *Sequoia sempervirens* (Lamb) Endl: A review. *Annals of Forest Science,* 50(3) 273-295.

Bairu, M.W., Aremu, A.O. and van Staden, J. (2011) Somaclonal variation in plants: causes and detection methods. *Plant Growth Regulation,* 63(2) 147-173.

Bairu, M.W. and Kane, M.E. (2011) Physiological and developmental problems encountered by *in vitro* cultured plants. *Plant Growth Regulation,* 63(2) 101-103.

Ballester, A. and Vieitez, A.M. (2012) Partial rejuvenation of mature hardwood trees through somatic embryogenesis: the example of pedunculate oak. In: Y. S. Park and J. M. Bonga (eds.) *Proceedings of the IUFRO Working Party 2.09.02 Conference on "Integrating Vegetative Propagation, Biotechnologies and Genetic Improvement for Tree Production and Sustainable Forest Management",* Brno, Czech Republic, 25-28 June 2012, 37-46.

Bausher, M.G. and Niedz, R.P. (1998) A discussion of *in vitro* contamination control of explants from greenhouse and field grown trees. *Proceedings – Florida State Horticultural Society,* 111 260-262.

Biroščíková, M., Spišáková, K., Lipták, Š., Pichler, V. and Ďurkovič, J. (2004) Micropropagation of mature wych elm (*Ulmus glabr* Huds.). *Plant Cell Reports,* 22(9) 640-644.

Bonga, J., Klimaszewska, K. and von Aderkas, P. (2010) Recalcitrance in clonal propagation, in particular of conifers. *Plant Cell, Tissue & Organ Culture,* 100(3) 241-254.

Bonga, J.M. (1987) Clonal propagation of mature trees: problems and possible solutions. In: J.M. Bonga and Don J. Durzan (eds.) *Cell and Tissue Culture in Forestry*. Springer Netherlands, 249-271.

Carroll, G. (1988) Fungal endophytes in stems and leaves: From latent pathogen to mutualistic symbiont. *Ecology,* 69(1) 2-9.

Cassells, A. and Curry, R. (2001) Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: Implications for micropropagators and genetic engineers. *Plant Cell, Tissue & Organ Culture*, 64(2) 145-157.

Chalupa, V. (1984) *In vitro* propagation of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). *Biologia Plantarum,* 26(5) 374-377.

Chalupa, V. (1987) Effect of benzylaminopurine and thidiazuron on *in vitro* shoot proliferation of *Tilia cordata* Mill., *Sorbus aucuparia* L. and *Robinia pseudoacaci* L. *Biologia Plantarum*, 29(6) 425-429.

Chalupa, V. (1990) Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). *Plant Cell Reports,* 9(7) 398-401.

Chalupa, V. (2003) *In vitro* propagation of *Tilia platyphyllos* by axillary shoot proliferation and somatic embryogenesis. *Journal of Forest Science,* 49(12) 537-543.

Clasen, T. and Edmondson, P. (2006) Sodium dichloroisocyanurate (NaDCC) tablets as an alternative to sodium hypochlorite for the routine treatment of drinking water at the household level. *International Journal of Hygiene and Environmental Health,* 209(2) 173-181.

Dar, C., Abdullah, J., Namasivayam P. and Roowi, S. (2012) Sterilization of *Hibiscus rosa-sinensis* L. vegetative explants sourced from plants grown in open environment and influences of organic ingredients on *in vitro* direct regeneration. *American Journal of Plant Sciences*, 3(6) 791-798.

Domir, S.C. and Schreiber, L.R. (1993) Effects of frequent subculturing of elm callus tissue on growth of *Ophiostoma ulmi*. *Journal of Aboriculture,* 19(1) 7-11.

Doronina, N.V., Ivanova, E.G., Suzina, N.E. and Trotsenko, Y.A. (2004) Methanotrophs and methylobacteria are found in woody plant tissues within the winter period. *Microbiology,* 73(6) 702-709.

Ďurkovič, J. and Mišalová, A. (2008) Micropropagation of temperate noble hardwoods: An overview. *Functional Plant Science and Biotechnology*, 2(1) 1-19.

Fry, D.J., Douglas, G.C. and Saieed, N.T. (1997) Somaclonal variation in *Populus*: An evaluation. *United States Department of Agriculture Forest Service General Technical Report RM,* (297) 33-43.

Gartland, K.M., Crow, R.M., Fenning, T.M. and Gartland, J.S., (2003) Genetically modified trees: production, properties, and potential. *Journal of Arboriculture*, *29*(5) 259-266.

Gartland, K.M., McHugh, A.T., Crow, R.M., Carg, A. and Gartland, J.S., (2005) 2004 SIVB Congress Symposium Proceeding: Biotechnological progress in dealing with Dutch elm disease. *In Vitro Cellular & Developmental Biology-Plant, 41*(4) 364-367.

Gaspar, T., Kevers, C., Penel, C., Greppin, H., Reid, D.M. and Thorpe, T.A. (1996) Plant hormones and plant growth regulators in plant tissue culture. *In Vitro Cellular and Developmental Biology-Plant,* 32(4) 272-289.

George, M.W. and Tripepi, R.R. (2001) Plant preservative mixture™ can affect shoot regeneration from leaf explants of chrysanthemum, European birch, and rhododendron. *HortScience,* 36(4) 768-769.

Gupta, P.K. and Durzan, D.J. (1987) Micropropagation and phase specificity in mature, elite Douglas fir. *Journal of the American Society for Horticultural Science,* 112 969-971.

Guri, A.Z. and Patel, K.N. (1998) *Compositions and methods to prevent microbial contamination of plant tissue culture media.* US5750402 A. United States. Available from https://docs.google.com/viewer?url=patentimages.storage.googleapis.com/pdfs/US5750402.pdf [Accessed 1 April 2015].

Hernández, I., Celestino, C. and Toribio, M. (2003) Vegetative propagation of Q*uercus suber* L. by somatic embryogenesis: I factors affecting the induction in leaves in mature cork oak trees. *Plant Cell Reports,* 21(8) 759-764.

Jones, O.P., Welander, M., Waller, B.J. and Ridout, M.S. (1996) Micropropagation of adult birch trees: production and field performance. *Tree Physiology*, 16(5) 521-525.

Kärkönen, A. (2000) Anatomical study of zygotic and somatic embryos of *Tilia cordata. Plant Cell, Tissue and Organ Culture,* 61(3) 205-214.

Larkin, P.J. and Scowcroft, W.R. (1981) Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theoretical and Applied Genetics,* 60(4) 197-214.

Leifert, C. and Cassells, A. (2001) Microbial hazards in plant tissue and cell cultures. *In Vitro Cellular and Developmental Biology - Plant,* 37(2) 133-138.

Lester, D.T. and Berbee, J.G. (1977) Within-clone variation among black poplar trees derived from callus culture. *Forest Science,* 23(1) 122-131.

Lineberger, R.D., Sticklen, M.B., Pijut, P.M., Kroggel, M.A., Fink, C.V.M. and Dormir, S.C. (1990) Use of protoplast, cell and shoot tip culture in an elm germplasm improvement program. *Acta Horticulturae,* 208 247-253.

Linsmaier, E.M. and Skoog, F. (1965) Organic growth factor requirements of tobacco tissue cultures. *Physiologia Plantarum,* 18(1) 100-127.

Lloyd, G. and McCown, B. (1980) Commercially feasible micropropagation of mountain laurel, *Kalimia latifolia*, by use of shoot-tip culture. *Proceedings of the International Plant Propagator's Society*, 30 421-427.

McCown, B. (2000) Recalcitrance of woody and herbaceous perennial plants: Dealing with genetic predeterminism. *In Vitro Cellular & Developmental Biology - Plant,* 36(3) 149-154.

Moubayidin, L., Di Mambro, R. and Sabatini, S. (2009) Cytokinin – auxin crosstalk. *Trends in Plant Science,* 14(10) 557-562.

Murashige, T. (1974) Plant propagation through tissue-cultures. *Annual Review of Plant Physiology,* 25 135-166.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, 15(3) 473-497.

Neelakandan, A. and Wang, K. (2012) Recent progress in the understanding of tissue culture-induced genome level changes in plants and potential applications. *Plant Cell Reports*, 31(4) 597-620.

Niedz, R.P. (1998) Isothiazolone biocides to control microbial and fungal contaminants in plant tissue cultures. *HortTechnology*, 8 598-601.

Niedz, R.P. and Bausher, M.G. (2002) Control of *in vitro* contamination of explants from greenhouse- and field-grown trees. *In Vitro Cellular & Developmental Biology-Plant*, 38(5) 468-471.

Novotny, D. (2004) Contribution to the knowledge tree tissue cultures associated mycobiota. *Acta Fytotechnica et Zootechnica*, 7 227-230.

Ostry, M., Hackett, W., Michler, C., Serres, R. and McCown, B. (1994) Influence of regeneration method and tissue source on the frequency of somatic variation in *Populus* to infection by *Septoria musiva. Plant Science*, 97(2) 209-215.

Parkinson, M., Prendergast, M. and Sayegh, A.J., 1996. Sterilisation of explants and cultures with sodium dichloroisocyanurate. *Plant Growth Regulation*, 20(1) 61-66.

Phillips, R.L., Kaepplert, S.M. and Olhoft, P. (1994) Genetic instability of plant tissue cultures: breakdown of normal controls. *Proceedings of the National Academy of Sciences of the USA*, 91 5222-5226.

Pigott, C.D. (1969) The status of *Tilia cordata* and *T. platyphyllos* on the Derbyshire limestone. *Journal of Ecology*, 57(2) 491-504.

Pigott, C.D. and Huntley, J.P. (1981) Factors controlling the distribution of *Tilia cordata* at the northern limits of its geographical range. III nature and causes of seed sterility. *New Phytologist*, 87(4) 817-839.

Pirttila, A.M., Pospiech, H., Laukkanen, H., Myllyla, R. and Hohtola, A. (2005) Seasonal variations in location and population structure of endophytes in buds of Scots pine. *Tree Physiology,* 25(3) 289-297.

Pirttilä, A.M., Pospiech, H., Laukkanen, H., Myllylä, R. and Hohtola, A. (2003) Two endophytic fungi in different tissues of Scots pine buds (*Pinus sylvestris* L.). *Microbial Ecology,* 45(1) 53-62.

Pirttilä, A.M. (2011) Endophytic bacteria in tree shoot tissues and their effects on host. In: A. C. Frank and Anna M. Pirttilä (eds.) *Endophytes of Forest Trees: biology and applications, Forestry sciences 80.* Springer, 139-149.

Quain, M.D., Berjak, P., Acheampong, E. and Kioko, J.I., (2009) Sucrose treatment and explant water content: critical factors to consider in development of successful cryopreservation protocols for shoot tip explants of the tropical species *Dioscorea rotundata* (yam). *CryoLetters,* 30(3) 212-223.

Ryan, R.P., Germaine, K., Franks, A., Ryan, D.J. and Dowling, D.N. (2008) Bacterial endophytes: Recent developments and applications. *Federation of European Microbiological Societies Microbiology Letters,* 278(1) 1-9.

Sanchez, M.C., San-Jose, M.C., Ballester, A. and Vieitez, A.M. (1996) Requirements for *in vitro* rooting of *Quercus robur* and *Q. rubra* shoots derived from mature trees. *Tree Physiology,* 16(8) 673-680.

Sanchez, M.C. and Vieitez, A.M. (1991) In vitro morphogenetic competence of basal sprouts and crown branches of mature chestnut. *Tree Physiology,* 8(1) 59-70.

Sarvašová, I. and Ďurkovič, J. (2002) *In v*itro regeneration of European linden. *Biologia Plantarum,* 45(1) 149-152.

Skirvin, R.M., McPheeters, K.D. and Norton, M. (1994) Sources and frequency of somaclonal variation. *Horticultural Science,* 29(11) 1232-1237.

Skoog, F. and Miller, C.O. (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symposia of the Society for Experimental Biology,* 11 118-130.

Smith, R.H. (2000) *Plant tissue culture techniques and experiments.* 2nd edition. Academic Press.

Smulders, M.J.M. and de Klerk, G.J. (2011) Epigenetics in plant tissue culture. *Plant Growth Regulation,* 63(2) 137-146.

Trotsenko, Y.A., Ivanova, E.G. and Doronina, N.V. (2001) Aerobic methylotrophic bacteria as phytosymbionts. *Microbiology* 70(6) 623-632.

Uchendu, E.E., Shukla, M.R., Reed, B.M. and Saxena, P.K., (2013) Melatonin enhances the recovery of cryopreserved shoot tips of American elm (*Ulmus americana* L.). *Journal of pineal research*, 55(4) 435-442.

Vijayan, K., Tikader, A. and Teixeira da Silva, J.A. (2011) Application of tissue culture techniques for propagation and crop improvement in mulberry (*Morus spp.*). *Tree and Forestry Science and Biotechnology,* 5 1-13.

Von Aderkas, P. and Bonga, J.M. (2000) Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. *Tree Physiology*, 20(14) 921-928.

Webster, C.A. and Jones, O.P. (1989) Micropropagation of the apple rootstock M.9 - effect of sustained subculture on apparent rejuvenation *in vitro*. *Journal of Horticultural Science,* 64(4) 421-428.

Zulfiqar, B., Abbasi, N.A., Ahmad, T. and Hafiz, I.A. (2009) Effect of explant sources and different concentrations of plant growth regulators on *in vitro* shoot proliferation and rooting of avocado (*Persea americana* Mill.) cv. "Fuerte". *Pakistan Journal of Botany,* 41(5) 2333-2346.

CHAPTER SIX

General Discussion

6.1 Discussion

The results reported in this thesis provide details of an investigation into the genetic diversity and structure of *Tilia cordata* Mill. within the Lincolnshire Limewoods in order to inform conservation strategies and replanting schemes for the woods. To facilitate this study, a *T. cordata* microsatellite library was constructed (chapter 3) and suitable loci were developed that would also amplify *T. platyphyllos* and, therefore, enable the identification of hybrid trees. The regenerative potential of *T. cordata* (chapter 5) was also investigated to assess the suitability of tissue culture techniques to provide planting material of local provenance that might be used in future woodland restoration schemes.

The trees in the Lincolnshire Limewoods are members of a woodland community that has been in existence for many thousands of years. Population fragmentation over many centuries, together with woodland management, may have affected the genetic structure of the trees in the woods, leaving them potentially more vulnerable to reduction in genetic variation and, therefore, less able to respond to changing environmental pressures. Trees from other similar ancient Limewoods from outside Lincolnshire were included in the study to investigate if the genetic diversity within the Lincolnshire Limewoods was typical of the diversity at a broader scale or whether the woods were under additional environmental pressures that might affect their diversity (chapter 4).

At the beginning of the study a cost effective method for DNA extraction was developed that would enable RAPD and microsatellite markers to be successfully amplified during PCR (chapter 2). A pilot study, using RAPD markers with *T. cordata* DNA, was undertaken to investigate the potential of using this marker for the population genetic study (chapter 2). RAPD was initially chosen as it has proved useful in studies into

species differentiation (Van de Ven. and McNicol, 1995; Nkongolo *et al*., 2002). It has been employed in population studies of a number of tree species, including *Tilia*, such as *Tilia rubra* (Hosseinzadeh Colagar *et al*., 2013), Douglas Fir (Aagaard *et al*., 1998) and wild service tree (Belletti *et al*., 2008). RAPD has also been used to detect clones and a study carried out by Cottrell *et al*. (1997) identified low genetic diversity and widespread clonal duplication within the British population of black poplar, recognising the endangered status of this native species. However, even though RAPD was able to identify individual *T. cordata*, it was considered that for a genetic population study, where the trees would be predominantly from one species and from closely grouped woods within Lincolnshire, extending the study with RAPD primers would produce less informative results than those obtained from codominant microsatellite markers. Nybom (2004) reports that, for plants, estimates of within-population diversity derived from microsatellite data are almost three times higher than those derived from dominant markers, while among-population diversity is generally similar.

Microsatellites have proved useful in many tree population studies, where they have been used to identify closely related genotypes and to investigate genetic diversity in natural and managed tree populations e.g. *Quercus petraea* L. (Muir *et al*., 2004), *Prunus avium* L. (Vaughan *et al*., 2007), *Fraxinus excelsior* L. (Provan *et al*., 2008). Since this research was primarily concerned with within-population diversity at a local scale, the utilisation of microsatellite markers was considered the most appropriate approach to determine the structure of the population of *T. cordata* in the Lincolnshire Limewoods. Microsatellite primers were not available for *T. cordata* at the beginning of the study and so a microsatellite library was constructed from which ten polymorphic loci were found to be suitable for the population study (chapter 3). These ten loci were able to

identify clones and could also amplify DNA from *T. platyphyllos* and were, therefore, used to detect hybridisation between *T. cordata* and *T. platyphyllos*.

Within the Lincolnshire Limewoods, *T. platyphyllos* and/or *T. cordata* x *T. platyphyllos* hybrids were identified in 11 of the 14 woods sampled and, although they are known to occur together in many British woods (Pigott, 1969; Logan *et al*., 2015), they had not previously been noted within the Lincolnshire Limewoods (chapter 4). Where *T. platyphyllos* and/or hybrid trees occurred within the woods, the genetic diversity of the woods was slightly, but significantly, higher than for the woods in the study where no *T. platyphyllos* were identified. More private alleles were also identified within these woods. An increase in diversity was also found to occur in *Ulmus Pumila* populations in woods where hybrids were found (Zalapa *et al*., 2010) and introgression increased diversity among yellow ladyslipper orchid populations (Klier *et al*., 1991). Alleles that are shared between *T. cordata* and *T. platyphyllos* (chapter 3) may be evidence of introgression between the two species (Baack and Rieseberg, 2007). For oak species, Muir and Schlotterer (2005) proposed that extensive gene sharing might result from shared ancestry. Within the Limewoods there may be evidence of both scenarios, with alleles that are extensively shared being evidence of shared ancestry and alleles shared at low frequency resulting from more recent introgression.

The populations sampled for this study were found, in general, to have similar levels of genetic diversity to that seen in other studies of *Tilia spp*., with *T. platyphyllos* more genetically diverse than *T. cordata* (Fromm and Hattemer, 2003; Hosseinzadeh Colagar *et al*., 2013; Phuekvilai and Wolff, 2013; Phuekvilai, 2014). As with many other tree species (e.g. *Fraxinus excelsior* (Beatty *et al*., 2015), *Quercus petraea* (Muir *et al*., 2004), *Pinus*

sylvestris L. (Provan *et al*., 1998)), most of the genetic variance is found within the individuals (82%), with little variation between the populations (4%). For *T. cordata*, small differences in the admixture distribution for the woods within the Lincolnshire Limewoods and the Forest of Bere, two geographically widely separated groups of trees, suggest that these areas may have differing genetic profiles which reflect an isolation-by-distance population structure. The Lincolnshire Limewoods should, therefore, be treated as a single population for management purposes.

From a conservation point of view, where hybridisation occurs between naturally occurring species, the hybrids should be considered to be an integral part of the ecosystem and their genetic diversity preserved (Whitham and Maschinski, 1996; Allendorf *et al*., 2001). However, hybridisation with plants that have been introduced to the woods is seen as less desirable, as it introduces genetic diversity that can compromise the historic diversity (Ellstrand and Schierenbeck, 2000). The origin of *T. platyphyllos* within the Lincolnshire Limewoods is not known. *T. platyphyllos* arrived in Britain with *T. cordata* at the end of the last glacial period (Mittre, 1971) but was also extensively planted, together with *T.* x *europaea*, in the 17th and 18th centuries (Pigott, 1992). The *T. platyphyllos* trees have a mean stool diameter of 2.1 m, suggesting that these trees are of considerable age and should be considered to be contributing to the biodiversity of the local ecosystem.

If all populations are genetically similar then replanting with seed from any wood might be perceived as being suitable for conservation and restoration planting schemes. However, neutral molecular markers such as microsatellites only inform about gene flow and migration and do not show how organisms are adapted to their environment. For the

Lincolnshire Limewoods, the most suitable policy for enabling expansion and regeneration within the woods would be to facilitate natural regeneration into adjacent areas and cleared conifer plantation sites. This should preserve the current biodiversity and genetic structure of the woods and will take into account the occurrence of *T. platyphyllos* and hybrid trees. For areas where natural regeneration is impractical, local *T. cordata* should be replanted. To increase the likelihood that only *T. cordata* is used, seeds should be taken from woods without hybrid populations and, preferably, should be screened to identify and exclude F1 hybrids.

At the start of the study there was little sexual regeneration within any of the Lincolnshire Limewoods. Tissue culture of *T. cordata* was considered as a potential supply of locally sourced plants that could be used in future schemes to enlarge the woods and increase their connectivity (chapter 5). Although successful tissue culture of *Tilia* spp. has been achieved (Kärkönen, 2000; Chalupa, 1990, 2003), in this study explant bacterial and fungal contamination, occurring after the initial surface sterilisation, was found to affect all cultures eventually. Factors such as the season in which the explants were collected and the treatment of the tissue prior to the removal of the explant material for sterilisation were found to be critical in reducing the effect of surface contamination on subsequent sterilisation procedures. As with *T. cordata*, the tissue culture of Douglas fir (Gupta and Durzan, 1987) was more successful when explants were collected in the spring. Allowing collected bud tissue to develop under clean conditions prior to explant selection was also effective in reducing contamination after sterilisation, as plants that are grown outdoors can harbour significantly greater amounts of endophytic organisms than plants grown in greenhouses (Smith, 2000; Vijayan *et al*., 2011). Where contamination was minimised, roots and shoots were produced but these cultures could not be maintained. Arnaud *et al*

(1993) suggests that successful tissue culture of mature plants is generally less successful than that derived from young tissue, since the ability of the vegetative tissues to differentiate into leaves, roots and stems declines with age; this may be one reason why the *T. cordata* tissue proved so recalcitrant. Strategies that improve the outcome of tissue culture include using juvenile tissue derived from seedlings and zygotic embryos from seeds, as successfully used by Kärkönen (2000) and Chalupa (1990). Locally sourced seedlings and rooted shoots from layers are also potential sources of planting stock where natural regeneration is not possible. Sourcing these locally will ensure that only plants that are adapted to local conditions are used and also reduce the possibility of introducing pathogens to which the trees have no resistance.

6.2 Further work

The presence of seedlings within the woods shows that locally collected seeds would be viable and could be used for new planting schemes. To further inform the choice of which populations to use, trials are required to identify the occurrence of inbreeding and the incidence of hybridisation, as well as to establish whether the genetic variation shown by the adult trees is reflected in the diversity of the seedlings. For use in further studies, a method of screening the seedlings to identify hybrids should be developed using the microsatellite markers designed for this study. Field trials are needed to investigate seedling survival rates and the incidence of seedling predation. Further investigation into the fine scale spatial structure within woods, with intensive sampling within forest coupes, would enable the extent of clonal populations and hybridisation to be determined. Over the long term, this study would allow naturally regenerating areas of the wood to be compared with managed coppiced woodlands and identify woodland areas where regeneration is occurring. The tissue culture of *T. cordata* might also be developed further

with the use of seeds as a source of zygotic embryos to produce somatic embryos (Chalupa, 2003).

6.3 Conclusion

This study has contributed to the knowledge of *T. cordata* and in particular to its status within the Lincolnshire Limewoods. The development of ten *T. cordata* microsatellite loci has facilitated the study and enabled the identification of *T. platyphyllos* and, for the first time, highlighted the presence of hybrid trees within the Lincolnshire Limewoods populations. For *T. cordata* there is evidence of hybridisation with *T. platyphyllos* and allele sharing between the two species suggests introgression has occurred. Within the Lincolnshire Limewoods *T. cordata* is not a rare plant and, despite fragmentation, the woods have maintained high levels of genetic diversity with differences between the woods being small. The expansion of the woodlands by natural regeneration is considered to be the most effective way of increasing biodiversity and reversing the effects of fragmentation (Peterken, 2002). Facilitating the expansion of the naturally occurring *T. cordata* into new areas, to expand the woods, will help to enhance the genetic fitness of the trees and leave them better able to adapt to environmental pressures such as global warming and pathogens.

6.4 References

Aagaard, J.E., Krutovskii, K.V. and Strauss, S.H. (1998) RAPD markers of mitochondrial origin exhibit lower population diversity and higher differentiation than RAPDs of nuclear origin in Douglas fir. *Molecular Ecology,* 7(7) 801-812.

Allendorf, F.W., Leary, R.F., Spruell, P. and Wenburg, J.K. (2001) The problems with hybrids: Setting conservation guidelines. *Trends in Ecology & Evolution,* 16(11) 613-622.

Arnaud, Y., Franclet, A., Tranvan, H. and Jacques, M. (1993) Micropropagation and rejuvenation of *Sequoia sempervirens* (Lamb) Endl: A review. *Annals of Forest Science.,* 50(3) 273-295.

Baack, E.J. and Rieseberg, L.H. (2007) A genomic view of introgression and hybrid speciation. *Current Opinion in Genetics & Development,* 17(6) 513-518.

Beatty, G.E., Brown, J.A., Cassidy, E.M., Finlay, C.M.V., McKendrick, L., Montgomery, W.I., Reid, N., Tosh, D.G. and Provan, J. (2015) Lack of genetic structure and evidence for long-distance dispersal in ash (*Fraxinus excelsio*r) populations under threat from an emergent fungal pathogen: Implications for restorative planting. *Tree Genetics & Genomes,* 11(3) 1-13.

Belletti, P., Monteleone, I. and Ferrazzini, D. (2008) A population genetic study in a scattered forest species, wild service tree (*Sorbus torminalis* (L.) Crantz)], using RAPD markers. *European Journal of Forest Research,* 127(2) 103-114.

Chalupa, V. (2003) *In vitro* propagation of *Tilia platyphyllos* by axillary shoot proliferation and somatic embryogenesis. *Journal of Forest Science,* 49(12) 537-543.

Chalupa, V. (1990) Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). *Plant Cell Reports,* 9(7) 398-401.

Cottrell, J.E., Forrest, G.I. and White, I.M.S. (1997) The use of RAPD analysis to study diversity in British black poplar (*Populus nigra* L. subsp. Betulifolia (Pursh) W. Wettst. (Salicaceae)) in Great Britain. *Watsonia*, 21(4) 305-312.

Ellstrand, N.C. and Schierenbeck, K.A. (2000) Hybridization as a stimulus for the evolution of invasiveness in plants? *Proceedings of the National Academy of Sciences of the United States of America*, 97(13) 7043-7050.

Fromm, M. and Hattemer, H.H. (2003) Inheritance of allozymes and hybridization in two european *Tilia* species. *Heredity*, 91(no 3, (September 1)) 337-344. Available from http://www.proquest.com/; Document ID: 868149881 [Accessed May 31, 2011].

Gupta, P.K. and Durzan, D.J. (1987) Micropropagation and phase specificity in mature, elite Douglas fir. *Journal of the American Society for Horticultural Science*, 112 969-971.

Hosseinzadeh Colagar, A., Yusefi, M., Zarei, M. and Yousefzadeh, H. (2013) Assessment of genetic diversity of *Tilia rubra* DC. by RAPD analysis in the Hyrcanian forests, north of Iran. *Polish Journal of .Ecoogyl*, 61(2) 341-348.

Kärkönen, A. (2000) Anatomical study of zygotic and somatic embryos of *Tilia cordata*.. *Plant Cell, Tissue and Organ Culture*, 61(3) 205-214.

Klier, K., Leoschke, M.J. and Wendel, J.F. (1991) Hybridization and introgression in white and yellow ladyslipper orchids (*Cypripedium candidum* and *C. pubescens*). *Journal of Heredity*, 82(4) 305-318.

Logan, S.A., Phuekvilai, P. and Wolff, K. (2015) Ancient woodlands in the limelight: Delineation and genetic structure of ancient woodland species *Tilia cordata* and *Tilia platyphyllos* (Tiliaceae) in the UK. *Tree Genetics & Genomes*, 11(3) 1-12.

Mittre, V. (1971) Fossil pollen of *Tilia* from the East Anglian fenland. *New Phytologist*, 70(4) 693-697.

Muir, G., Lowe, A.J., Fleming, C.C. and Vogl, C. (2004) High nuclear genetic diversity, high levels of outcrossing and low differentiation among remnant populations of *Quercus petraea* at the margin of its range in Ireland. *Annals of Botany*, 93(6) 691-697.

Muir, G. and Schloetterer, C. (2005) Evidence for shared ancestral polymorphism rather than recurrent gene flow at microsatellite loci differentiating two hybridizing oaks (*Quercus spp*.). *Molecular Ecology,* 14(2) 549-561.

Nkongolo, K.K., Michael, P. and Gratton, W.S. (2002) Identification and characterization of RAPD markers inferring genetic relationships among pine species. *Genome,* 45(1) 51-58.

Nybom, H. (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Molecular Ecology,* 13(5) 1143-1155.

Peterken, G.F. (2002) *Reversing the habitat fragmentation of British woodlands.* World Wildlife Fund-UK.

Phuekvilai, P. (2014) *Relicts, refugia and reticulation: a study of population history, hybrids and phylogeny in the long-lived flowering tree genus Tilia.* PhD Newcastle University.

Phuekvilai, P. and Wolff, K. (2013) Characterization of microsatellite loci in *Tilia platyphyllos* (Malvaceae) and cross-amplification in related species. *Applications in Plant Sciences,* 1(4) 1200386.

Pigott, C.D. (1969) The status of *Tilia cordata* and *T. platyphyllos* on the Derbyshire limestone. *Journal of Ecology,* 57(2) 491-504.

Pigott, D. (1992) The clones of common lime (*Tilia*×*vulgaris* Hayne) planted in England during the seventeenth and eighteenth centuries. *New Phytologist,* 121(3) 487-493.

Provan, J., Beatty, G.E., Hunter, A.M., McDonald, R.A., McLaughlin, E., Preston, S.J. and Wilson, S. (2008) Restricted gene flow in fragmented populations of a wind-pollinated tree. *Conservation Genetics,* 9(6) 1521-1532.

Provan, J., Soranzo, N., Wilson, N.J., McNicol, J.W., Forrest, G.I., Cottrell, J. and Powell, W. (1998) Gene-pool variation in Caledonian and European Scots pine (*Pinus sylvestris* L.) revealed by chloroplast simple-sequence repeats. *Proceedings.Biological Sciences / the Royal Society,* 265(1407) 1697-1705.

Smith, R.H. (2000) *Plant tissue culture techniques and experiments.* 2nd edition. Academic Press.

Van de Ven, Wilhelmina T.G. and McNicol, R.J. (1995) The use of RAPD markers for the identification of Sitka spruce (*Picea sitchensis)* clones. *Heridity,* 75 126-126.

Vaughan, S.P., Cottrell, J.E., Moodley, D.J., Connolly, T. and Russell, K. (2007) Distribution and fine-scale spatial-genetic structure in British wild cherry (*Prunus avium* L.). *Heredity*, 98(5) 274-283.

Vijayan, K., Tikader, A. and Teixeira da Silva, J.A. (2011) Application of tissue culture techniques for propagation and crop improvement in mulberry (*Morus spp.*). *Tree and Forestry Science and Biotechnology,* 5 1-13.

Whitham, T.G. and Maschinski, J. (1996) Current hybrid policy and the importance of hybrid plants in conservation. In: *Southwestern Rare and Endangered Plants: Proceedings of the Second Conference. General Technical Report RM-283. US Forest Service, Rocky Mountain Forest and Range Experiment Station, Ft. Collins, Colorado*, 103-112.

Zalapa, J.E., Brunet, J. and Guries, R.P. (2010) The extent of hybridization and its impact on the genetic diversity and population structure of an invasive tree, *Ulmus pumila* (Ulmaceae). *Evolutionary Applications,* 3(2) 157-168.

Appendices

Appendix 1

Maps of the General Location of all Woods Sampled and Maps of Individual Woods Showing Individual Trees with their Genotype Assignment.

Maps of the General Location of all Woods Sampled and Maps of Individual Woods Showing Individual Trees with their Genotype Assignment.

Table showing the location of the woods from which tissue samples were obtained.....250

Map of the general location of all woods sampled in England. 251

Location of all sampled woods within Lincolnshire. 252

The Chambers Farm group of woods. 253

Woods from the Bardney Limewoods: 254

Scotgrove Wood from the Bardney Limewoods. 255

Southrey Wood from the Bardney Limewoods. 256

Rand Wood from the Bardney Limewoods. 257

Wickenby Wood from the Bardney Limewoods. 258

Potterhanworth Wood (Lincolnshire). 259

Skellingthorpe Old Wood (Lincolnshire).....260

Woods from the Forest of Bere. 261

Hurst Wood from the Forest of Bere.....262

Vantage Copse and Bottom Copse from the Forest of Bere. 263

Welshbury Wood (Gloucestershire). 264

King's Wood (Buckinghamshire). 265

Spring Park (GLC). 266

Snipes Dene (Tyne and Wear). 266

T. platyphyllos from Wenlock Edge (Shropshire).....267

T. platyphyllos from Riseholme Park, Lincolnshire (a planted avenue). 267

Key for individual genotype assignment.

- *Tilia cordata*
- *Tilia platyphyllos*
- F1 hybrid
- F2 hybrid

Table showing the location of the woods from which tissue samples were obtained. The wood group and the regional identity (R) of the woods are also indicated.

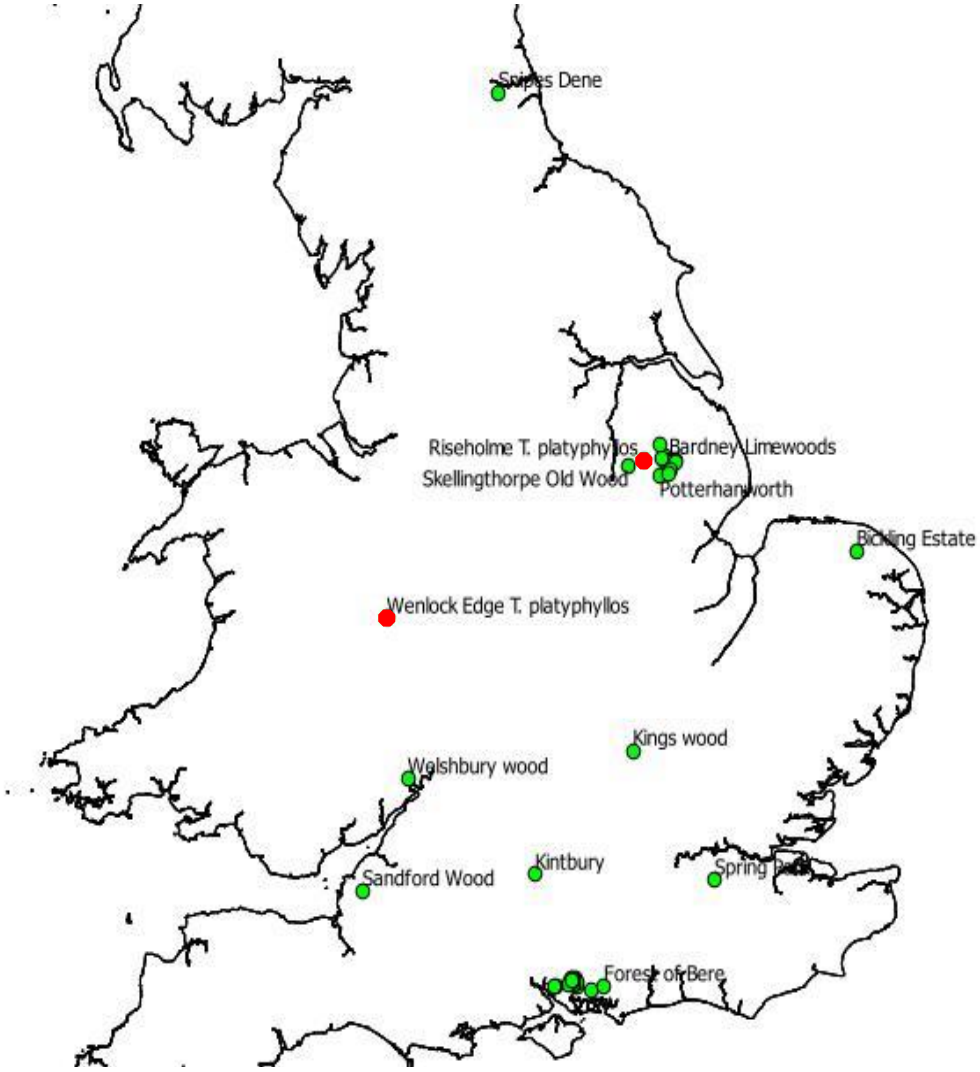
Wood name		Wood group	R	County	No. trees collected	Latitude	Longitude
Great Scrubbs	*	Bardney L'woods	L	Lincolnshire	25	53.25357	-0.27876
Hatton Wood	*	Bardney L'woods	L	Lincolnshire	18	53.25993	-0.25726
Ivy Wood	*	Bardney L'woods	L	Lincolnshire	31	53.24873	-0.28543
Little Scrubbs Wood	*	Bardney L'woods	L	Lincolnshire	20	53.25407	-0.28808
Minting Wood		Bardney L'woods	L	Lincolnshire	6	53.25279	-0.26011
Cocklode Wood	*	Bardney L'woods	L	Lincolnshire	30	53.27606	-0.34617
College Wood	*	Bardney L'woods	L	Lincolnshire	20	53.26477	-0.31374
Goslings Corner Wood	*	Bardney L'woods	L	Lincolnshire	20	53.26273	-0.29019
Hardy Gang Wood	*	Bardney L'woods	L	Lincolnshire	20	53.25913	-0.35774
Newball Wood	*	Bardney L'woods	L	Lincolnshire	20	53.27048	-0.36716
Rand Wood		Bardney L'woods	L	Lincolnshire	5	53.28903	-0.38030
Scotgrove Wood	*	Bardney L'woods	L	Lincolnshire	20	53.21993	-0.30792
Southrey Wood	*	Bardney L'woods	L	Lincolnshire	20	53.19956	-0.31349
Wickenby Wood	*	Bardney L'woods	L	Lincolnshire	21	53.33368	-0.38096
Potterhanworth Wood	*		L	Lincolnshire	20	53.18877	-0.39523
Skellingthorpe Old Wood	*		L	Lincolnshire	19	53.23772	-0.65162
Bottom Copse		Forest of Bere	S	Hampshire	2	50.8959	-1.29454
Vantage Copse		Forest of Bere	S	Hampshire	5	50.89898	-1.29791
Abbots Hill Wood	*†	Forest of Bere	S	Hampshire	3	50.90518	-1.13668
Bere Copse		Forest of Bere	S	Hampshire	2	50.93039	-1.14048
Bishops Wood		Forest of Bere	S	Hampshire	1	50.92108	-1.17138
Gardiners Purlieu		Forest of Bere	S	Hampshire	2	50.90432	-1.14238
Goat House Wood		Forest of Bere	S	Hampshire	2	50.8971	-1.13825
Hipley Copse		Forest of Bere	S	Hampshire	1	50.8966	-1.11963
Huntbourne Wood		Forest of Bere	S	Hampshire	7	50.91224	-1.11806
Lymington Purlieu	*	Forest of Bere	S	Hampshire	7	50.91243	-1.14366
Mill Copse		Forest of Bere	S	Hampshire	2	50.92683	-1.14624
West Walk	*†	Forest of Bere	S	Hampshire	4	50.91251	-1.15503
W. Wickham relic tree		Forest of Bere	S	Hampshire	1	50.90821	-1.19650
Coach Road Copse		Forest of Bere	S	Hampshire	1	50.93251	-1.16051
Great Lion Copse	*	Forest of Bere	S	Hampshire	10	50.9308	-1.16011
Huntage Copse	*	Forest of Bere	S	Hampshire	10	50.9248	-1.16292
Hurst Wood		Forest of Bere	S	Hampshire	5	50.88173	-1.01486
Stanstead Wood			S	Hampshire	2	50.89614	-0.91502
Bickling Estate				Norfolk	4	52.81962	1.20860
Kintbury			C	Berkshire	8	51.39585	-1.373250
Kings Wood			C	Buckinghamshire	6	51.95699	-0.65481
Spring Park	*		C	GLC	17	51.36293	-0.01264
Sandford Wood	*		W	Somerset	17	51.32827	-2.82112
Welshbury Wood	*		W	Gloucestershire	18	51.83602	-2.46777
Snipes Dene				Tyne and Wear	11	54.92896	-1.71909
Wenlock Edge		*T. platyphyllos*		Shropshire	7	52.56552	-2.63728

* Woods with ten or more trees sampled, used for population study.

† Adjacent wood, samples amalgamated with Lymington Purlieu to form >10 tree group.

R Region: L - Lincolnshire, S - South, C - Central, W - West

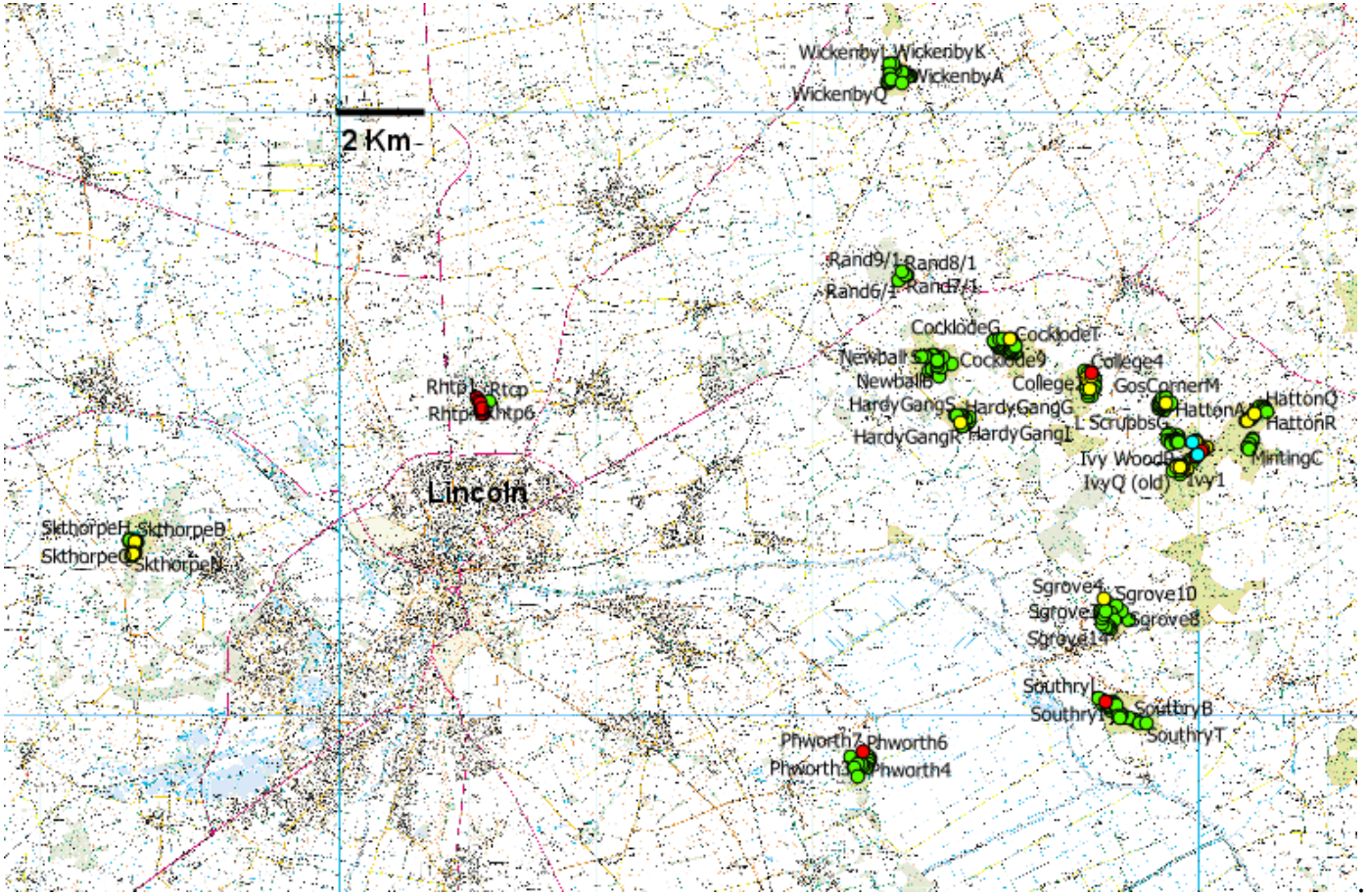
Map of the general location of all woods sampled in England.



- *T. cordata* populations
- *T. platyphyllos* populations

(Ordnance Survey, 2014a)

Location of all sampled woods within Lincolnshire.

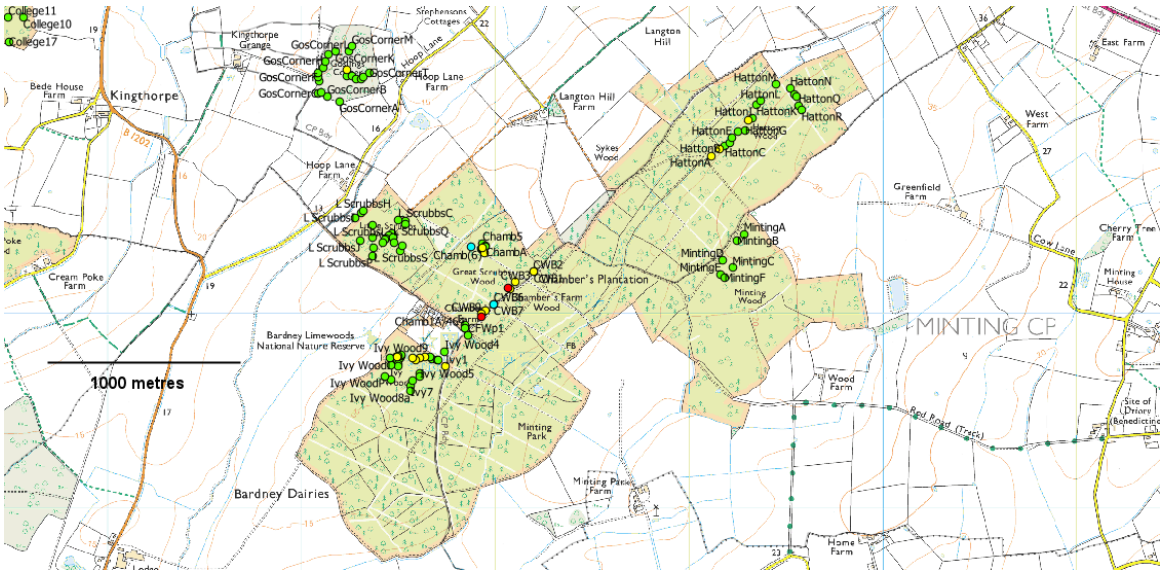


© Crown Copyright/database right 2014. An Ordnance Survey/EDINA supplied service

(Ordnance Survey, 2014b; Ordnance Survey, 2014d)

The Chambers Farm group of woods.

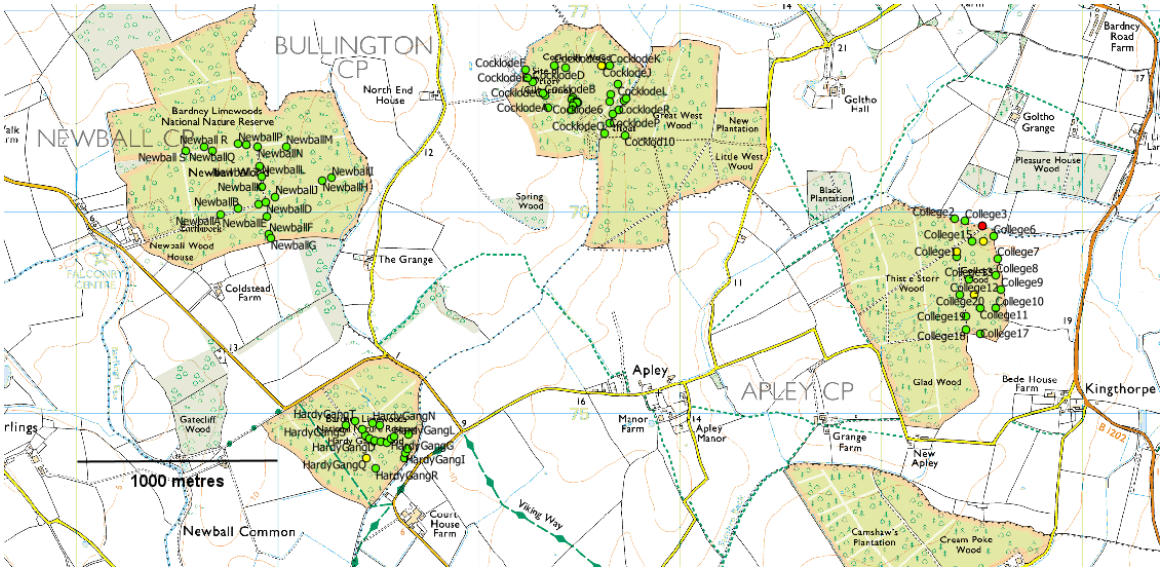
Included in this group are Great Scrubbs Wood, Little Scrubbs Wood, Hatton Wood, Minting Wood and Ivy Wood. Also shown is the nearby Goslings Corner Wood. These are all members of the Bardney Limewoods.



(Ordnance Survey, 2014d)

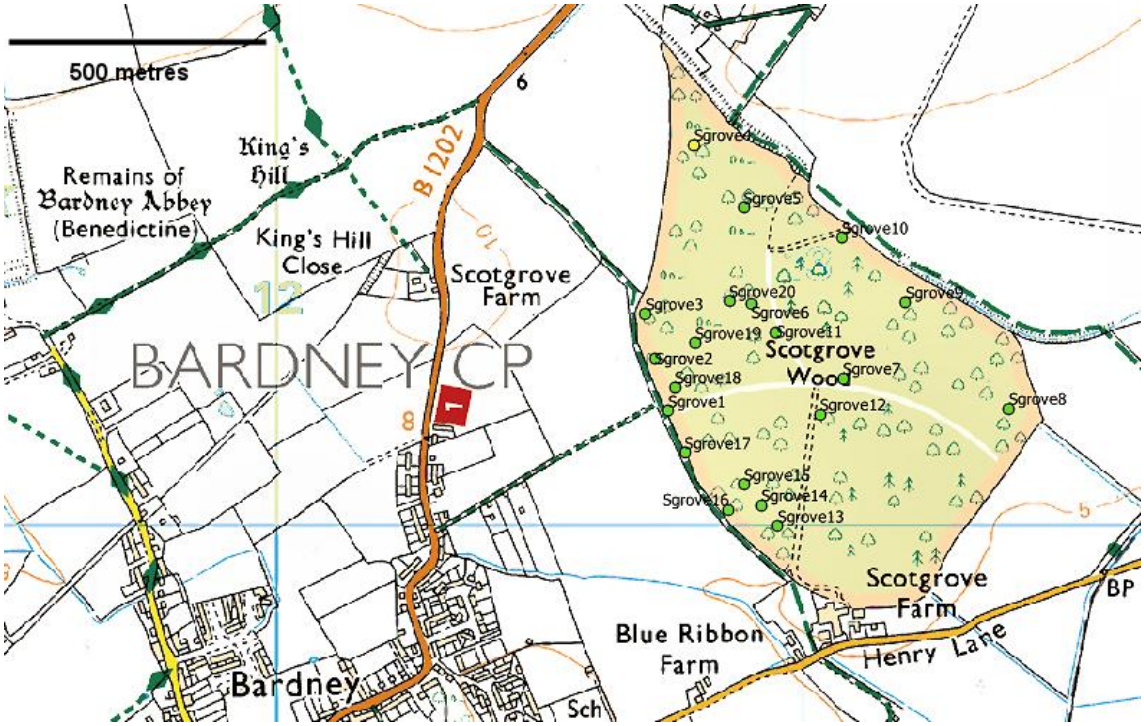
Woods from the Bardney Limewoods:

Newball Wood, Cocklode and Great West Wood, College Wood and Hardy Gang Wood.



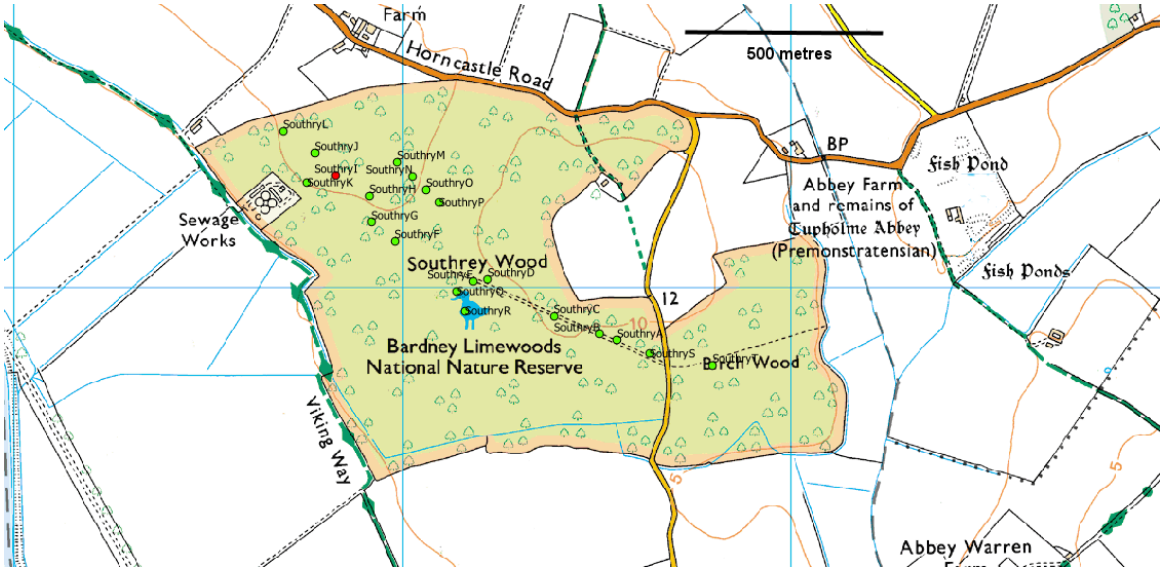
(Ordnance Survey, 2014d)

Scotgrove Wood from the Bardney Limewoods.



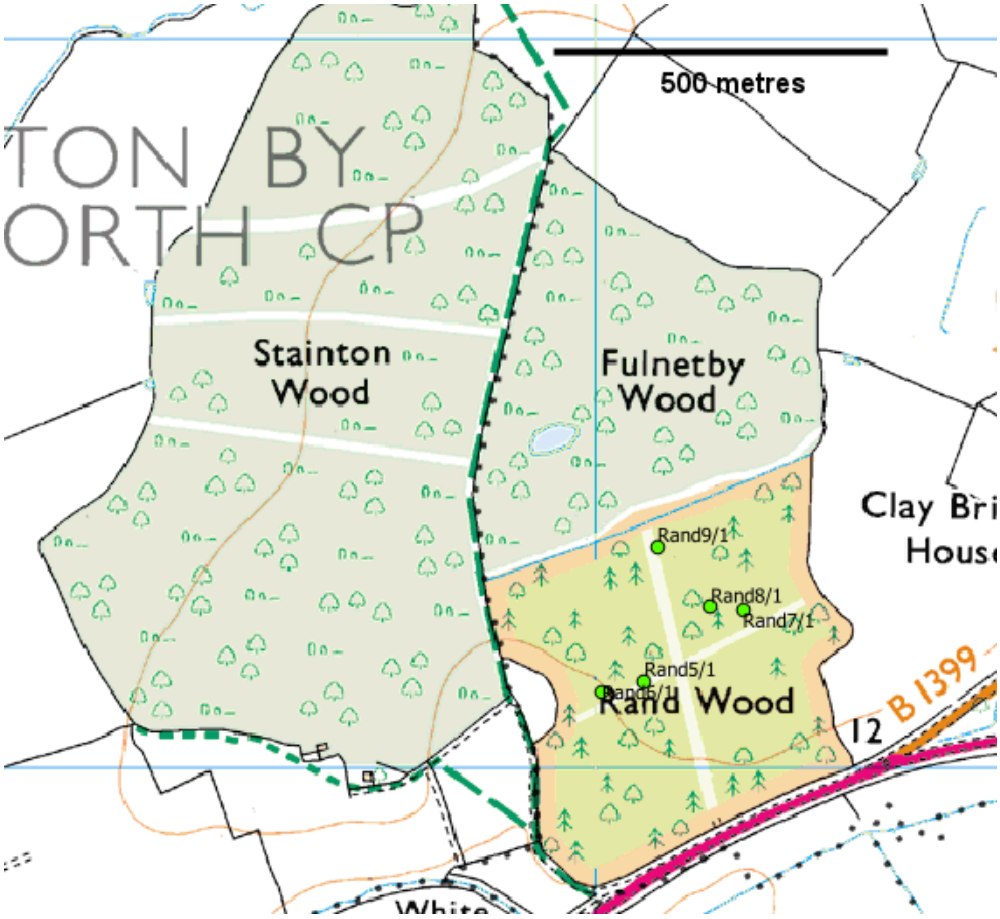
(Ordnance Survey, 2014d)

Southrey Wood from the Bardney Limewoods.



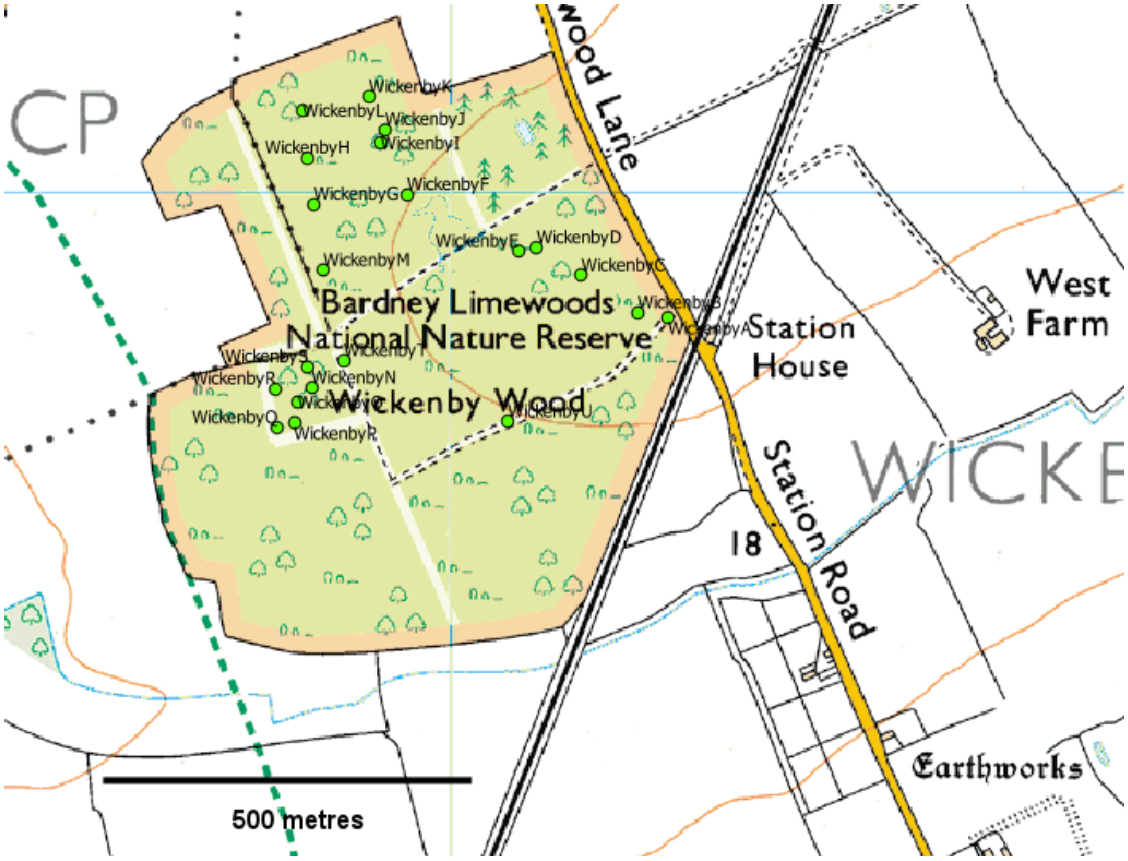
(Ordnance Survey, 2014d)

Rand Wood from the Bardney Limewoods.



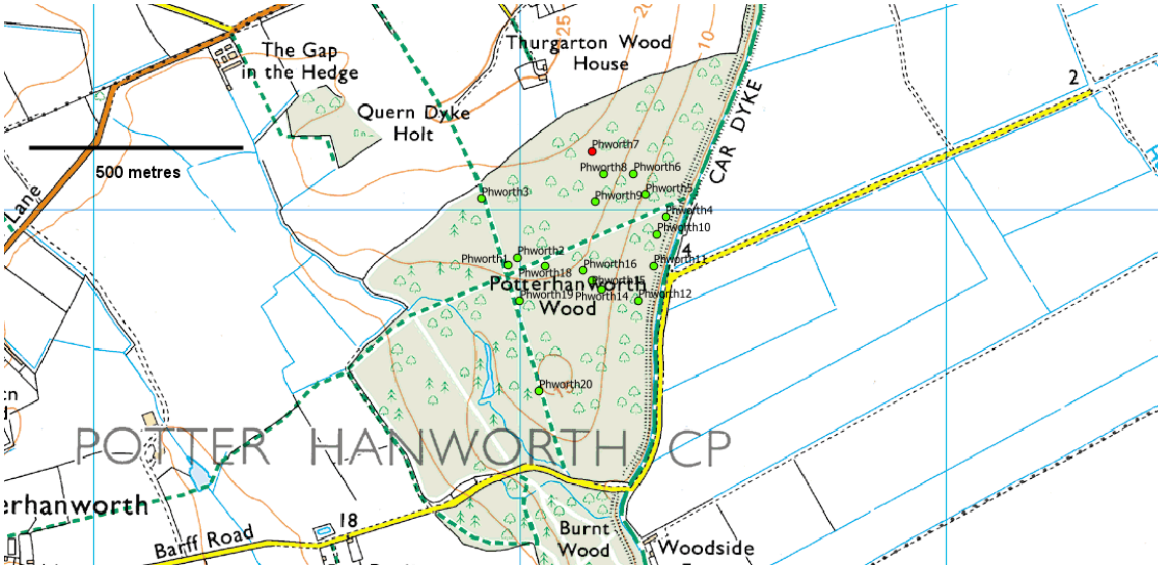
(Ordnance Survey, 2014d)

Wickenby Wood from the Bardney Limewoods.



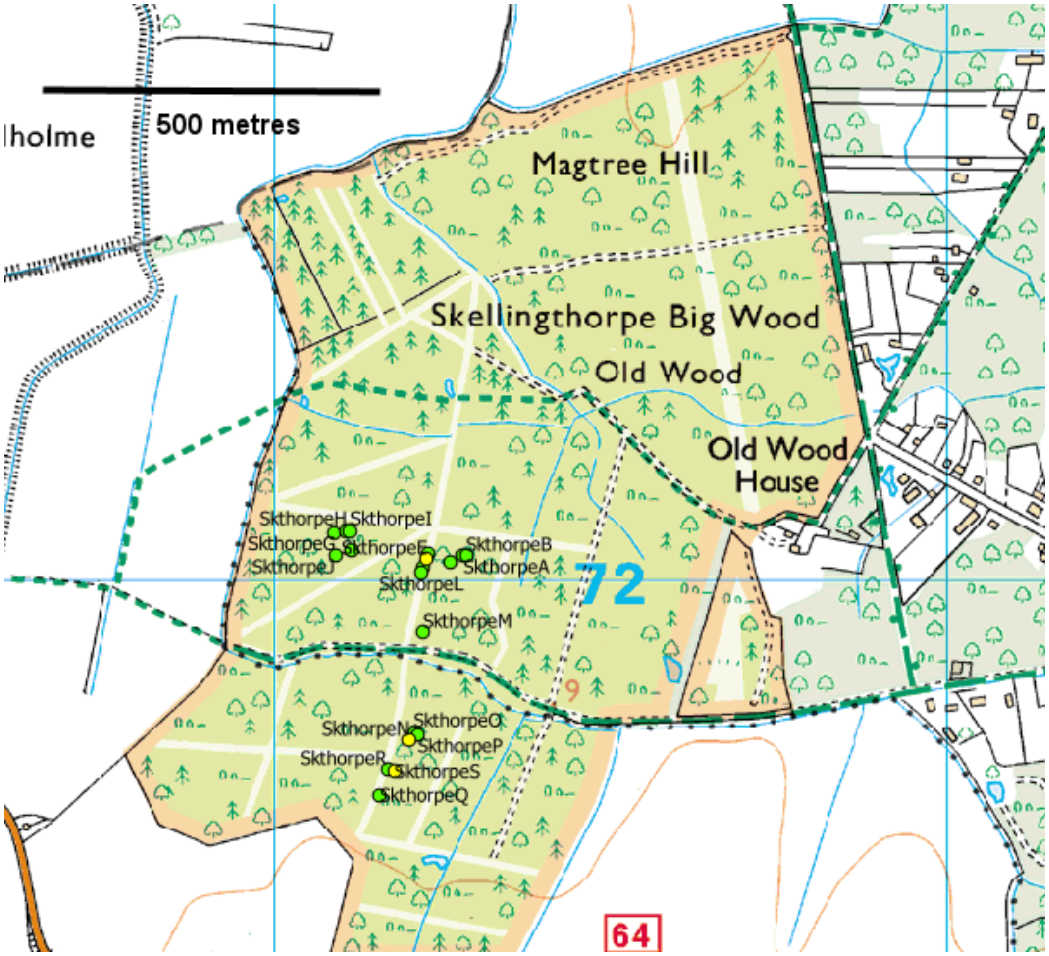
(Ordnance Survey, 2014d)

Potterhanworth Wood (Lincolnshire).



(Ordnance Survey, 2014d)

Skellingthorpe Old Wood (Lincolnshire).

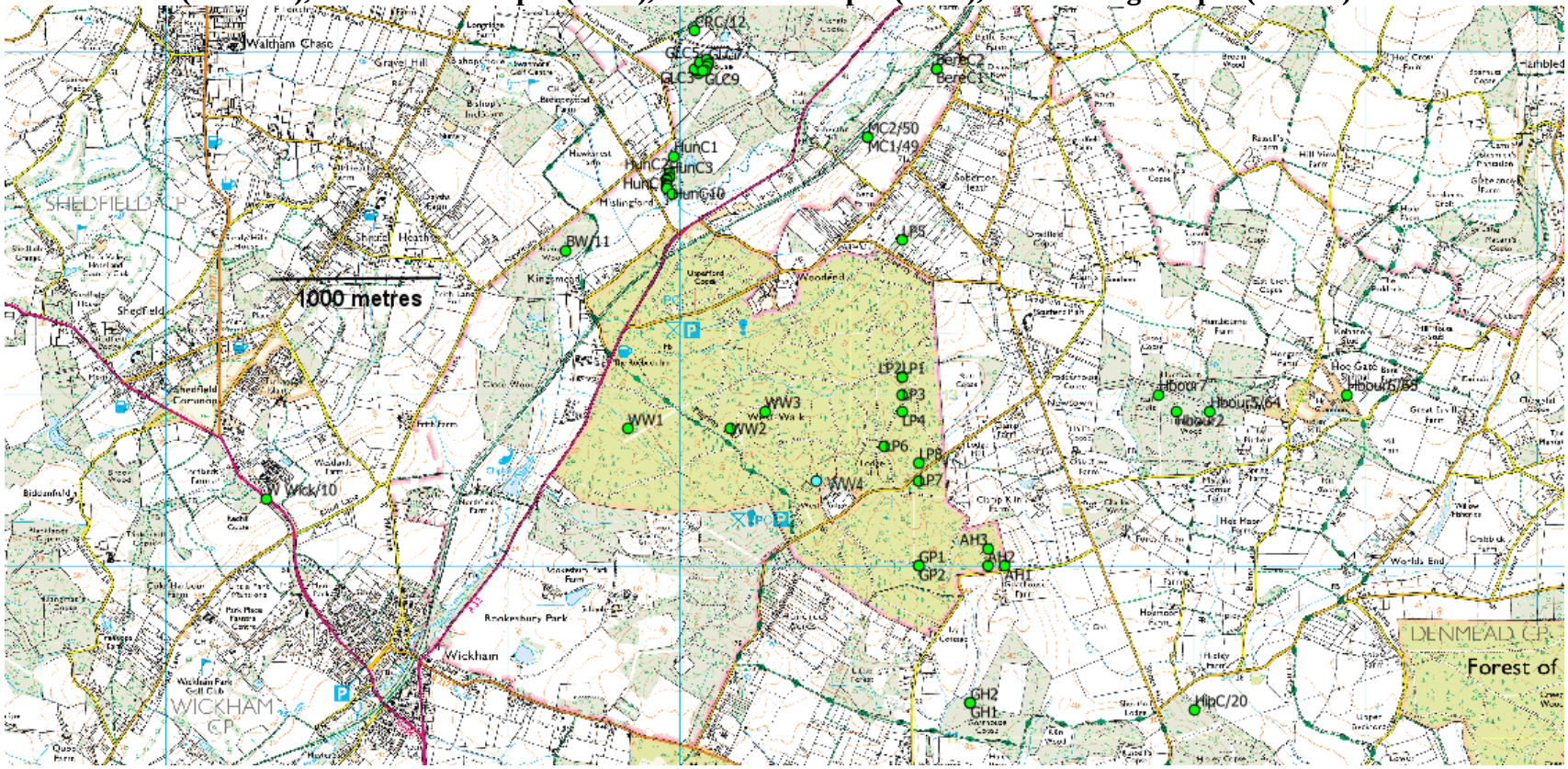


(Ordnance Survey, 2014b)

.

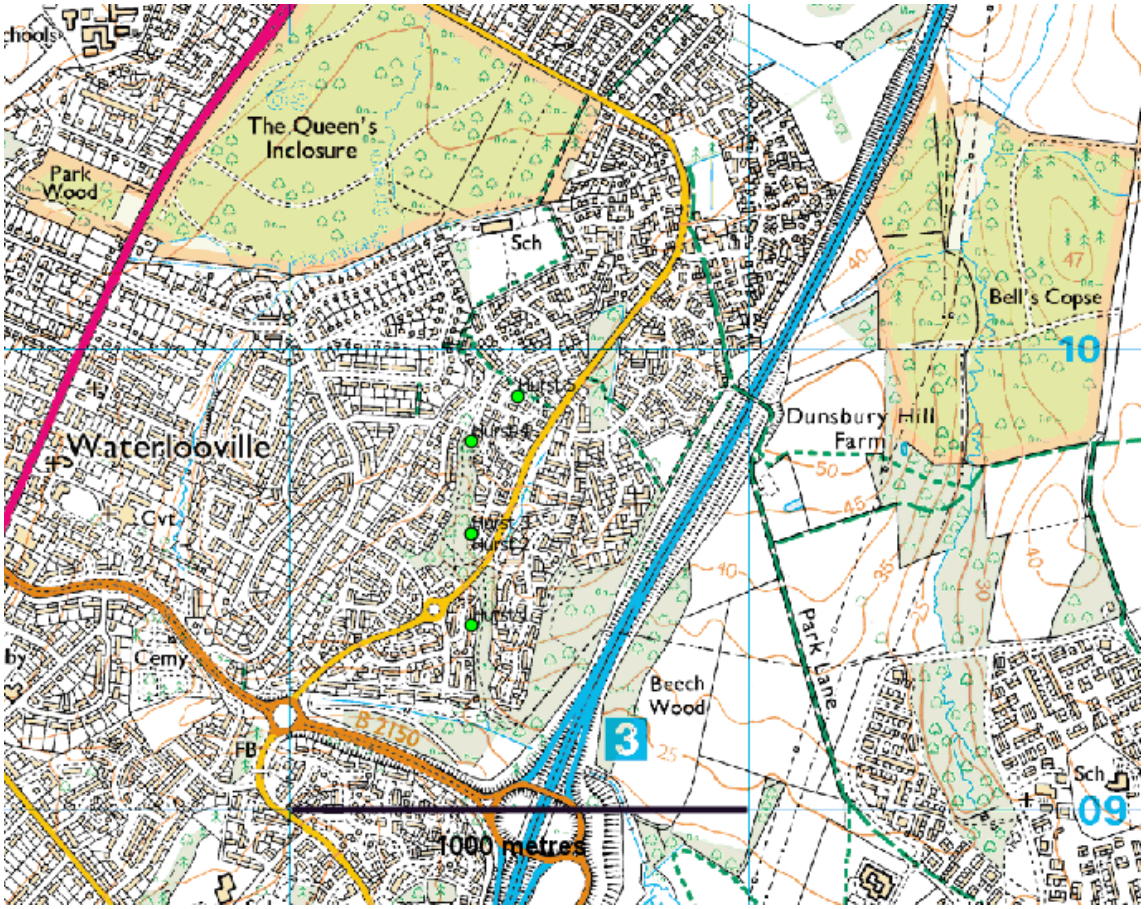
Woods from the Forest of Bere.

Abbots Hill (AH), Bere Copse (BC), Bishops Wood (BW), Gardiners Purlieu (GP), Hipley Copse (HipC), Huntbourne Copse (Hbourn), Lymington Purlieu (LP), Mill Copse (MC), West Walk (WW) (the location of the only non *T. cordata* tree in this forest group), West Wickham-relic tree (W Wick), Coach Road Copse (CRC), Great Lion Copse (GLC), and Huntage Copse (HuntC).



(Ordnance Survey, 2014d).

Hurst Wood from the Forest of Bere.

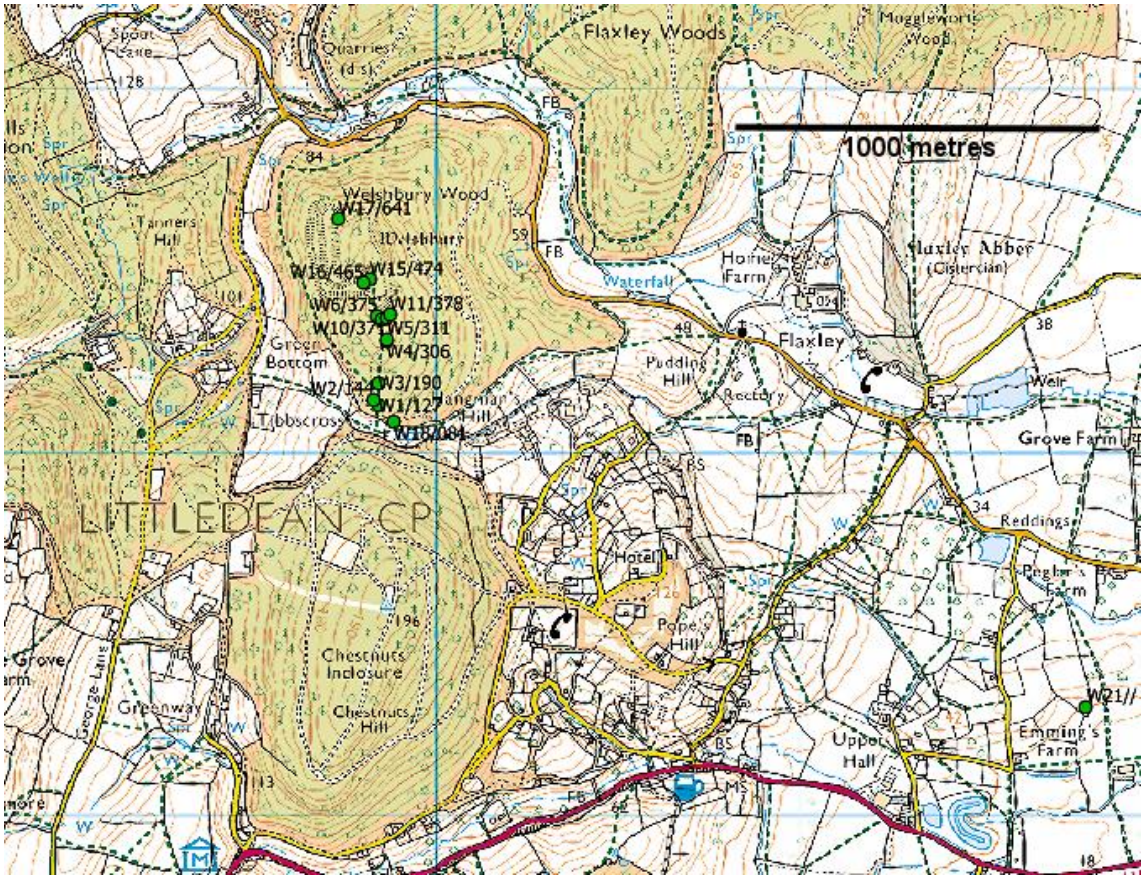


(Ordnance Survey, 2014c)

Vantage Copse and Bottom Copse from the Forest of Bere.

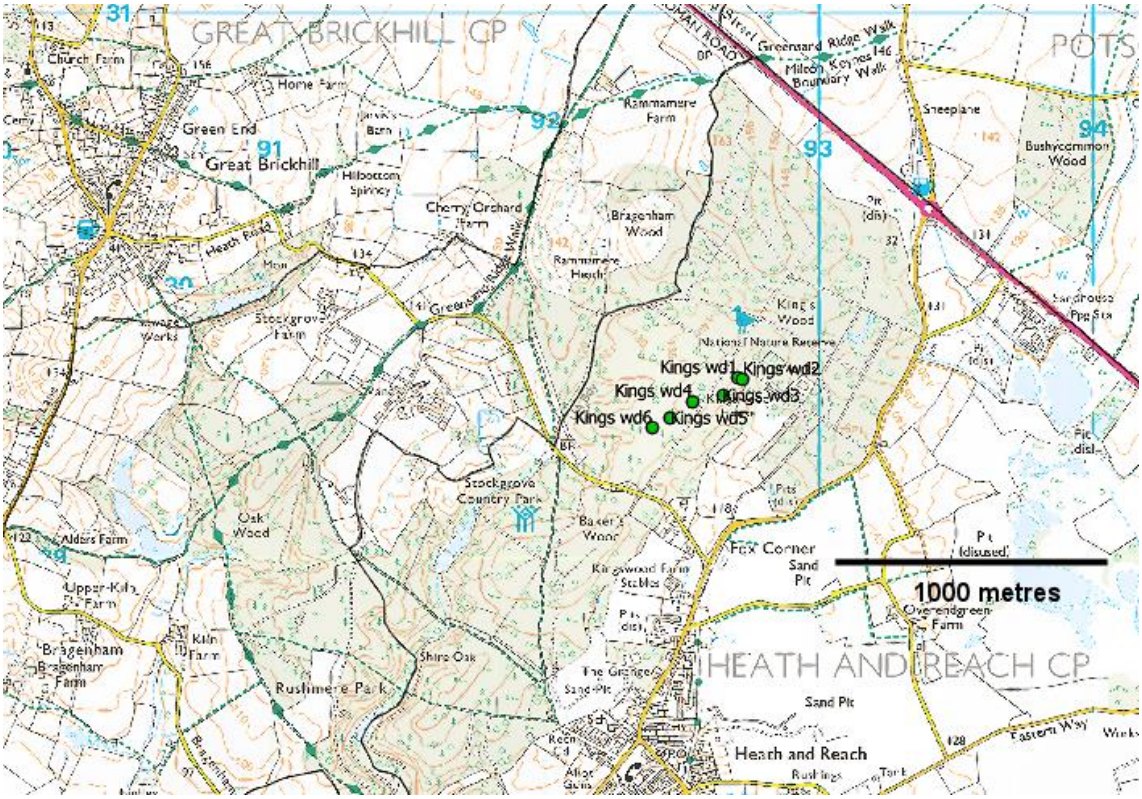
(Ordnance Survey, 2014c)

Welshbury Wood (Gloucestershire).



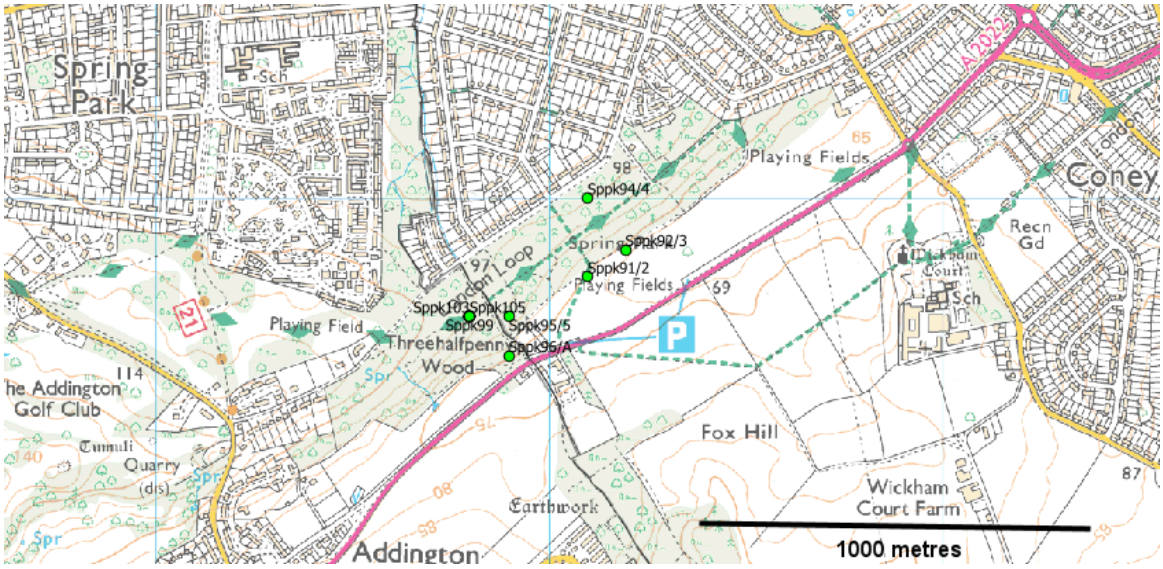
(Ordnance Survey, 2014k)

King's Wood (Buckinghamshire).



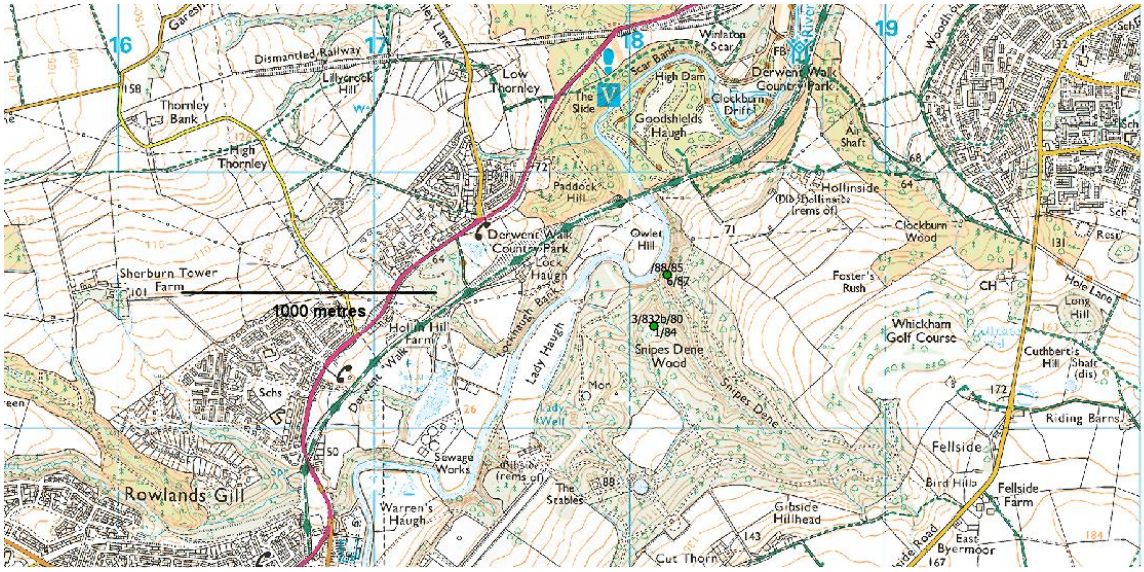
. © Crown Copyright/database right 2014. An Ordnance Survey/EDINA supplied service.
(Ordnance Survey, 2014i)

Spring Park (GLC).



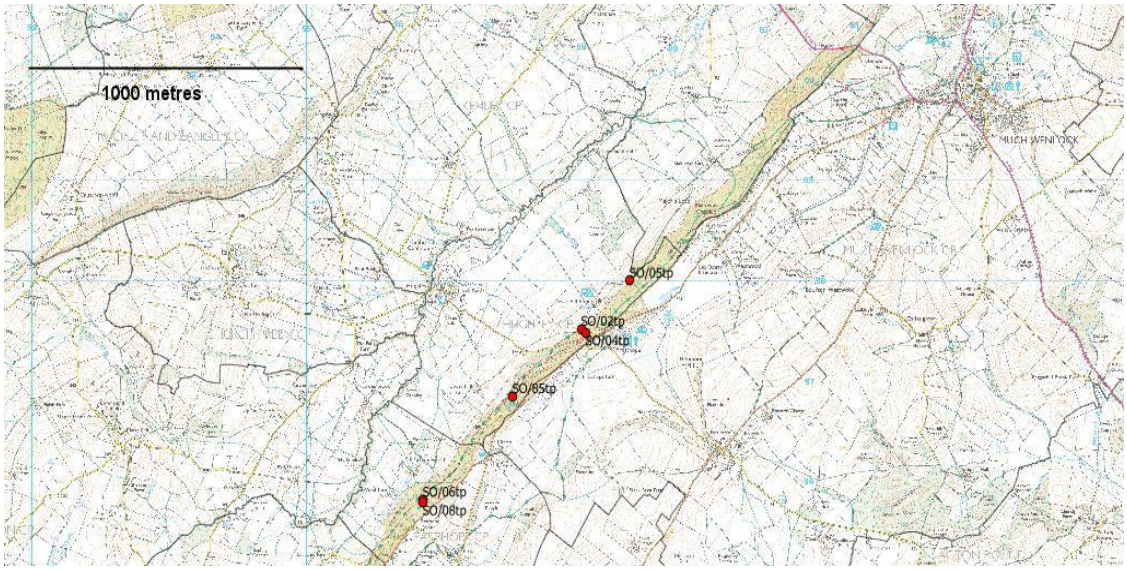
© Crown Copyright/database right 2014. An Ordnance Survey/EDINA supplied service.
(Ordnance Survey, 2014f)

Snipes Dene (Tyne and Wear).



. © Crown Copyright/database right 2014. An Ordnance Survey/EDINA supplied service.
(Ordnance Survey, 2014e)

***T. platyphyllos* from Wenlock Edge (Shropshire)**



(Ordnance Survey, 2014k; Ordnance Survey, 2014l)

***T. platyphyllos* from Riseholme Park, Lincolnshire (a planted avenue).**

(Ordnance Survey, 2014d)

References

Ordnance Survey (2014a) *Boundary Line: High Water [Shape geospatial data], Coverage: Great Britain.* [map], 1:10000 Ordnance Survey (GB). Available from EDINA Digimap Ordnance Survey Service, <http://digimap.edina.ac.uk; [Accessed 26/08/14].

Ordnance Survey (2014b) *1:25 000 Raster [TIFF geospatial data], tiles: SK86, SK87, SK88,SK96, SK97, SK98.* [map], 1:25000 Ordnance Survey (GB). Available from EDINA Digimap Ordnance Survey Service, <http://digimap.edina.ac.uk; [Accessed 27/08/14].

Ordnance Survey (2014c) *1:25 000 Raster [TIFF geospatial data], tiles: SU41, SU50, SU51, SU60, SU61, SU70, SU71.* [map], 1:25000 Ordnance Survey (GB). Available from EDINA Digimap Ordnance Survey Service, <http://digimap.edina.ac.uk; [Accessed 27/08/14].

Ordnance Survey (2014d) *1:25 000 Raster [TIFF geospatial data], tiles: TF06, TF07, TF08, TF16, TF17, TF18.* [map], 1:25000 Ordnance Survey (GB). Available from EDINA Digimap Ordnance Survey Service, <http://digimap.edina.ac.uk; [Accessed 27/08/14].

Ordnance Survey (2014e) *1:25 000 Raster [TIFF geospatial data], tiles: NZ15, NZ16.* [map], 1:25000 Ordnance Survey (GB). Available from EDINA Digimap Ordnance Survey Service, <http://digimap.edina.ac.uk; [Accessed 27/08/14].

Ordnance Survey (2014f) *1:25 000 Raster [TIFF geospatial data], tiles: TQ36.* [map], 1:25000 Ordnance Survey (GB). Available from EDINA Digimap Ordnance Survey Service, <http://digimap.edina.ac.uk; [Accessed 27/08/14].

Ordnance Survey (2014i) *1:25 000 Raster [TIFF geospatial data], tiles: SP92, SP93.* [map], 1:25000 Ordnance Survey (GB). Available from EDINA Digimap Ordnance Survey Service, <http://digimap.edina.ac.uk; [Accessed 27/08/14].

Ordnance Survey (2014k) *1:25 000 Raster [TIFF geospatial data], tiles: SO61.* [map], 1:25000 Ordnance Survey (GB). Available from EDINA Digimap Ordnance Survey Service, <http://digimap.edina.ac.uk; [Accessed 27/08/14].

Ordnance Survey (2014l) *1:25 000 Raster [TIFF geospatial data], tiles: SJ50, SJ60, SO59, SO69.* [map], 1:25000 Ordnance Survey (GB). Available from EDINA Digimap Ordnance Survey Service, <http://digimap.edina.ac.uk [Accessed 27/08/14].

Appendix 2

Pairwise Fst derived from AMOVA for: -

A. 20 wood populations with *T. cordata* trees

B. 21 wood populations with *T. cordata, T. platyphyllos* and hybrid trees.

Appendix 2A Pairwise Fst derived from AMOVA for 20 wood populations with *T. cordata* trees

Significant values of F_{ST} are shown in bold. Non-significant F_{ST}, where p values are >0.05 (based on 10^4 permutations) is shown in grey.

	GtSbs	Hton	Ivy	LtSbs	Clode	Coll	GsCr	HGng	Nball	Sgrve	Sthrey	Wkby	Pwrth	Sthpe	LymP	GtLn	HunC	SpPk	Sford	Wbry
GtScrubbs																				
Hatton	**0.032**																			
Ivy	**0.042**	0.003																		
LtScrubbs	**0.038**	0.016	**0.014**																	
Cocklode	**0.050**	0.011	0.000	0.006																
College	**0.033**	**0.036**	**0.038**	**0.028**	**0.042**															
GCorner	**0.038**	0.017	**0.015**	0.004	**0.019**	0.003														
HGang	**0.045**	0.009	0.002	0.000	0.005	**0.037**	0.002													
Newball	**0.037**	**0.039**	**0.023**	**0.024**	**0.033**	**0.020**	**0.023**	**0.025**												
Scotgrove	**0.039**	**0.025**	**0.034**	**0.012**	**0.033**	0.008	**0.030**	**0.018**	**0.026**											
Southrey	**0.036**	**0.001**	**0.025**	**0.029**	**0.014**	**0.038**	**0.034**	**0.027**	**0.053**	**0.029**										
Wickenby	**0.049**	0.011	0.004	**0.021**	0.000	**0.044**	**0.034**	**0.011**	**0.041**	**0.043**	**0.014**									
Phanworth	**0.036**	0.002	**0.017**	0.014	**0.019**	0.013	0.000	0.013	0.015	**0.019**	**0.031**	**0.035**								
Skthorpe	**0.047**	0.019	0.014	0.008	**0.024**	**0.041**	0.005	0.007	**0.027**	**0.049**	**0.052**	**0.030**	0.004							
LymP	**0.076**	**0.035**	**0.034**	**0.061**	**0.045**	**0.057**	**0.036**	**0.051**	**0.076**	**0.080**	**0.046**	**0.060**	**0.021**	**0.043**						
GtLion	**0.070**	**0.052**	**0.085**	**0.065**	**0.080**	**0.086**	**0.073**	**0.060**	**0.101**	**0.076**	**0.057**	**0.099**	**0.061**	**0.072**	**0.065**					
HunC	**0.103**	**0.042**	**0.067**	**0.073**	**0.058**	**0.093**	**0.072**	**0.045**	**0.103**	**0.079**	**0.051**	**0.073**	**0.039**	**0.048**	0.020	**0.029**				
SpPark	**0.151**	**0.157**	**0.152**	**0.146**	**0.155**	**0.131**	**0.112**	**0.159**	**0.167**	**0.185**	**0.153**	**0.180**	**0.109**	**0.143**	**0.090**	**0.111**	**0.158**			
Sandford	**0.087**	**0.038**	**0.051**	**0.049**	**0.073**	**0.069**	**0.043**	**0.045**	**0.078**	**0.088**	**0.097**	**0.084**	**0.041**	**0.025**	**0.065**	**0.092**	**0.103**	**0.164**		
Welshbury	**0.039**	**0.041**	**0.033**	0.016	**0.038**	**0.031**	**0.022**	**0.020**	**0.041**	**0.050**	**0.058**	**0.041**	0.018	0.020	**0.051**	**0.060**	**0.078**	**0.086**	**0.029**	

Appendix 2B Pairwise Fst derived from AMOVA for 21 wood populations with *T. cordata, T. platyphyllos* and hybrid trees.

Significant values of F_{ST} are shown in bold. Non-significant F_{ST}, where p values are >0.05 (based on 10^4 permutations) is shown in grey.

	GtSbs	Hton	Ivy	LtSbs	Clode	Coll	GsCr	HGng	Nball	Sgrve	Sthrey	Wkby	Pwrth	Sthpe	LymP	GtLn	HunC	SpPk	Sford	Wbry	tp
GtScrubbs																					
Hatton	**0.034**																				
Ivy	**0.033**	0.002																			
LtScrubbs	**0.076**	**0.021**	**0.026**																		
Cocklode	**0.070**	0.012	0.009	0.006																	
College	**0.029**	**0.022**	**0.025**	**0.033**	**0.037**																
GCorner	**0.066**	**0.016**	**0.016**	0.003	**0.015**	0.009															
HGang	**0.062**	0.012	0.006	0.000	0.002	**0.030**	0.002														
Newball	**0.083**	**0.044**	**0.030**	**0.024**	**0.030**	**0.030**	**0.021**	**0.028**													
Scotgrove	**0.064**	**0.023**	**0.034**	0.010	**0.027**	0.007	**0.022**	**0.017**	**0.021**												
Southrey	**0.055**	0.003	**0.030**	**0.029**	**0.012**	**0.023**	**0.030**	**0.025**	**0.049**	**0.020**											
Wickenby	**0.078**	**0.017**	**0.018**	**0.021**	0.000	**0.045**	**0.031**	**0.009**	**0.041**	**0.039**	**0.014**										
Phanworth	**0.052**	0.005	**0.017**	**0.016**	**0.018**	0.006	0.000	**0.016**	0.010	0.008	**0.023**	**0.034**									
Skthorpe	**0.052**	0.007	0.007	0.012	**0.017**	0.029	0.003	0.006	**0.032**	**0.036**	**0.040**	**0.025**	0.008								
LymP	**0.062**	**0.027**	**0.028**	**0.057**	**0.041**	**0.035**	**0.030**	**0.041**	**0.071**	**0.069**	**0.041**	**0.058**	0.013	**0.034**							
GtLion	**0.082**	**0.052**	**0.085**	**0.065**	**0.076**	**0.067**	**0.068**	**0.058**	**0.101**	**0.070**	**0.052**	**0.099**	**0.057**	**0.066**	**0.064**						
HunC	**0.090**	**0.035**	**0.065**	**0.073**	**0.055**	**0.066**	**0.064**	**0.039**	**0.103**	**0.073**	**0.046**	**0.073**	**0.035**	**0.040**	0.025	0.029					
SpPark	**0.149**	**0.153**	**0.148**	**0.146**	**0.152**	**0.120**	**0.115**	**0.159**	**0.167**	**0.176**	**0.147**	**0.180**	**0.106**	**0.143**	**0.089**	**0.111**	**0.158**				
Sandford	**0.102**	**0.046**	**0.059**	**0.049**	**0.072**	**0.065**	**0.043**	**0.044**	**0.078**	**0.082**	**0.091**	**0.084**	**0.042**	**0.032**	**0.063**	**0.092**	**0.103**	**0.164**			
Welshbury	**0.073**	**0.051**	**0.044**	0.016	**0.038**	**0.035**	**0.024**	**0.021**	**0.041**	**0.045**	**0.054**	**0.041**	**0.018**	**0.029**	**0.047**	**0.060**	**0.078**	**0.086**	**0.029**		
tp (Rh+WE)	**0.154**	**0.291**	**0.290**	**0.371**	**0.356**	**0.266**	**0.353**	**0.343**	**0.347**	**0.326**	**0.320**	**0.372**	**0.312**	**0.329**	**0.328**	**0.344**	**0.353**	**0.426**	**0.387**	**0.362**	

Appendix 3

Tissue Culture Media Composition and Comparison

For the pilot study of this investigation, the medium of choice was that developed by Murashige and Skoog in 1962 for the propagation of tobacco tissue cultures. This widely-used plant tissue culture medium is characterised by high concentrations of nitrate, potassium and ammonium salts, as compared to other media, such as Gamborg's B5 medium (Gamborg *et al*., 1968) and McCowan's woody plant medium (Lloyd and McCown, 1980). In subsequent trials, woody plant medium was also used as a low salt medium for the propagation of shoots from axillary buds of *T. cordata* (Chalupa, 1984).

The table below shows the composition of these three media and allows comparison between them.

Plant Tissue Culture Media Composition (mg/litre)

	Murashige and Skoog (MS)	Woody Plant Media (WPM)	B5 (Gamborg)
Ammonium nitrate	1650.0	400.0	
Ammonium sulphate			134.0
Boric acid	6.20	6.20	3.00
Calcium chloride.$2H_2O$	440.0	96.0	150.0
Calcium nitrate.$4H_2O$		556.0	
Cobalt chloride.$6H_2O$	0.025		0.025
Cupric sulphate.$5H_2O$	0.025	0.025	0.025
Magnesium sulphate	370.0	370.0	250.0
Manganese sulphate.H_2O	16.90	22.30	10.00
Potassium iodide	0.83		0.75
Potassium nitrate	1900.0		2500.0
Potassium phosphate	170.0	170.0	
Potassium sulphate		990.0	
Sodium molybdate.$2H_2O$	0.25	0.03	0.25
Sodium phosphate.H_2O			150.0
Zinc sulphate.$7H_2O$	8.60	8.60	2.00
Ferrous sulphate.$7H_2O$	27.80	27.80	27.80
Sodium EDTA	37.30	37.30	37.30
Myo-inositol	100.0	100.0	100.0
Nicotinic acid	0.50	0.50	1.00
Pyridoxine HCl	0.50	0.50	1.00
Thiamine HCl	0.40	1.00	10.00
Glycine		2.00	
Sucrose	3.0%	2.0%	3.0%
Agar	0.8%	0.6%	

Note: Plant growth regulators (auxins and cytokinins) are also added as required.

References

Chalupa, V. (1984) *Invitro* propagation of oak (*quercus robur* L) and linden (*Tilia cordata* Mill). *Biologia Plantarum*, 26(5) 374-377.

Gamborg, O.L., Miller, R.A. and Ojima, K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, 50(1) 151-158.

Lloyd, G. and McCown, B. (1980) Commercially feasible micropropagation of mountain laurel, *Kalimia latifolia*, by use of shoot-tip culture. *Proceedings of the Iinternational Plant Propagator's Society*, 30 421-427.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, 15(3) 473-497.